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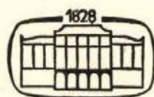
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L. KESZTYŰS, I. NÁSZ, K. RAUSS, F. B. STRAUB, L. VÁCZI,
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TOMUS XXII

FASCICULUS I



AKADÉMIAI KIADÓ, BUDAPEST

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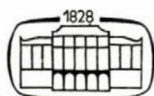
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BONE CHANGES IN YOUNG MICE WITH IMPAIRED LYMPHOID SYSTEM*

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(Received July 19, 1973)

Summary. Disturbances of osseal growth were observed in young mice with their lymphoid system affected by antithymocyte serum or mitolactol (dibromodulcitol) treatment. These bone changes were similar to those observed in germ-free and neonatally thymectomized mice as well as in mice suffering from a graft vs. host reaction. Their severity was in direct correlation with the disturbance of the thymus dependent lymphoid system. Not only immunological adaptation but also normal bone growth appears to require an intact thymus and thymus dependent lymphoid system.

Thymectomy in neonatal mice was found to cause besides its well-known consequences, disturbances in osseal growth. A retardation of enchondral ossification, weakness of the femoral cortical plate as well as changes in intercellular substance formation in the growth plates have been observed [1-3].

Examinations were then made on the skeletal system on young germ-free mice. Owing to the lack of antigenic stimuli, the thymus dependent lymphoid system of these animals was underdeveloped and showed a decreased function as compared to the controls. They displayed a retardation of growth and of enchondral ossification. The bone changes observed in neonatally thymectomized and in germ-free mice were qualitatively similar and were assumed to be associated with the lacking function of the thymus and thymus dependent lymphocytes [4]. In addition, we observed a retardation of enchondral ossification in mice with graft vs. host reaction (GVH) [5].

The present study has therefore been devoted to the question of whether bone changes were demonstrable in mice with their lymphoid system affected by different procedures such as antithymocyte serum (ATS) or mitolactol treatment.

Materials and methods

Animals. Inbred C3H mice were used in both experiments.

Antithymocyte serum treatment. (a) ATS was produced by immunizing rabbits with the thymus cell suspension of young C3H mice. GRAY's technique [6] was applied for preparation of the cell suspension, immunization and absorption of serum with mouse erythrocytes.

(b) The effect of ATS *in vitro* was studied by leuco-agglutination. Cell suspension of a C3H mouse thymus served as antigen. The agglutinin titre of ATS was 1 : 512 as determined

* Presented at the Annual Meeting of the Hungarian Association of Immunologists Budapest 1972.

with TAKÁTSY's micromethod [7]. The ATS effect *in vivo* was studied in young C3H mice. Four hours after a single intraperitoneal injection of 0.5 ml of ATS diluted 1 : 1 with physiological saline, the mean absolute lymphocyte count in peripheral blood dropped from 3000 to 500. On the next day it rose to 900, but did not reach the mean value of the controls even on the 14th day.

(c) Three to four weeks old C3H mice were subjected to ATS treatment. The animals were given intraperitoneally 0.5 ml serum diluted with an equal amount of physiological saline every other day. The treatment was repeated on 7 occasions. Animals of the same litter were used for control and were given physiological saline instead of ATS.

Mitolactol treatment. (a) Mitolactol (dibromodulcitol, Chinoin, Hungary) is an alkylating cytostatic with a lymphotropic effect [8, 9]. The substance was suspended in sterile physiological saline and 5% (v/v). Tween 80 were added. The dilution used for intraperitoneal injection was 0.01 ml/g body weight. The LD₅₀/21 days for mice was 600 mg/kg as determined after a single treatment.

(b) Four-weeks-old C3H mice were injected with 1/10 LD₅₀ of mitolactol (ML) daily on 11 occasions. Animals of the same litter were used for control.

Absolute lymphocyte count was estimated under standardized conditions in blood samples taken from the tail vein.

Study of the skeletal system. Radiographs were taken and radiomicrometric measurements were made under identical conditions on pairs of treated and control animals. The method of examination has been described earlier [2].

Histological examinations were performed on the preparations of the distal end of the femur of both treated and control mice, in each experiment. The specimens were fixed in formalin, decalcified and embedded in paraffin. Sections were stained with haema oxylin-eosin and alcian-blue PAS.

Results

1. **ATS treated mice.** Examinations were performed on 12 ATS-treated and 12 control mice. They were sacrificed 5 days after the last treatment, at 6 weeks of age.

ATS treatment reduced markedly the number of circulating lymphocytes. On the fifth day after the last treatment the average decrease was 85% as compared to the controls. The average loss of body weight amounted to 9% (Fig. 1).

According to radiomicrometric measurements, the retardation of both longitudinal and diametric growth amounted to 8%. The decrease of cortical thickness was more marked: 28% (Fig. 2).

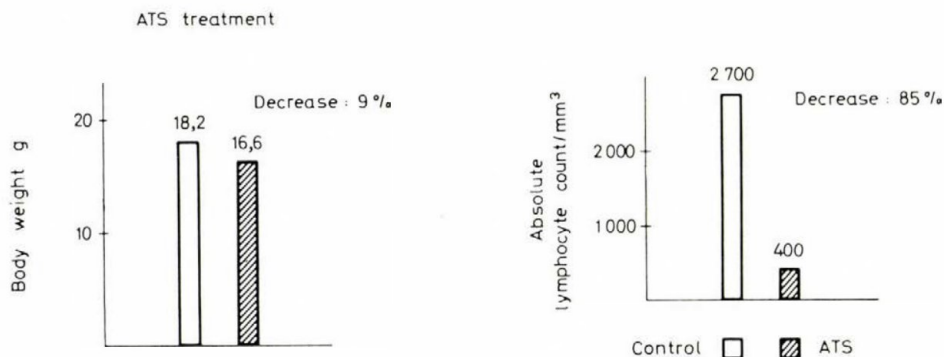


Fig. 1. Mean body weight and absolute lymphocyte count of ATS-treated and control mice

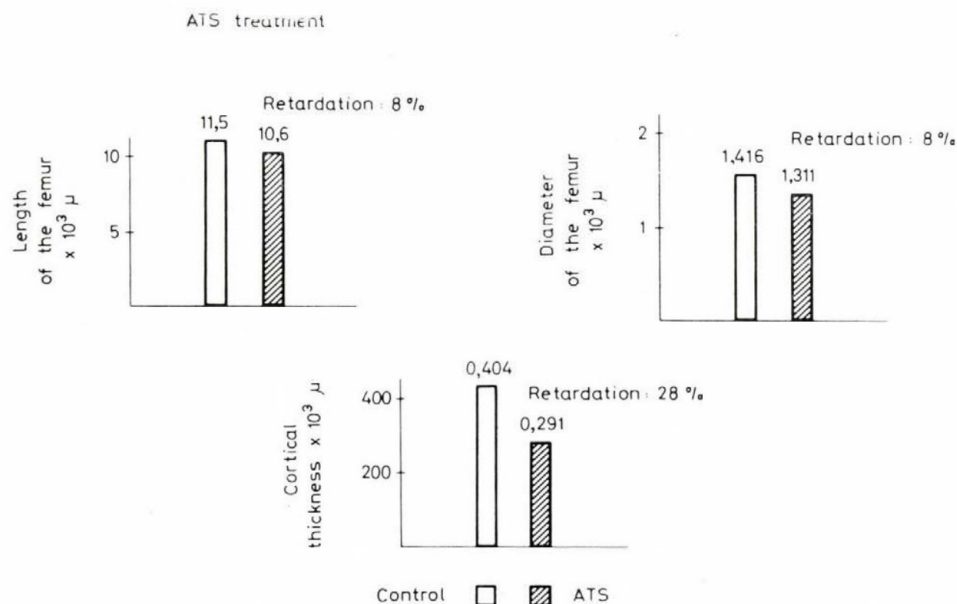


Fig. 2. Mean radiomicrometric measurements of ATS-treated and control mice

The distal femoral epiphysis of control animals displayed normal endochondral ossification (Fig. 3a).

In the distal end of the femur, a reduction in number and thinning of spongy trabeculae was observed. Growth plates were smaller and the number of cells was low in each cell layer. Hyaline pillars stagnated mostly in the cartilaginous state, with little osteoblastic activity on their edges. The picture indicated retarded endochondral ossification (Fig. 3b).

Alcian blue-PAS staining revealed alcianophilic intercellular substance in the growth plates of control animals, while that of the ATS-treated animals displayed PAS positivity, indicating an altered matrix formation.

2. *ML-treated mice.* Fourteen ML-treated and 12 control animals were examined. They were sacrificed 5 days after the last treatment at the age of 6 weeks. On the fifth day after the last treatment the decrease of absolute lymphocyte count was 66% and the loss of body weight 21% as compared to the controls (Fig. 4).

Radiomicrometric measurements revealed 11% longitudinal and 8% diametric growth retardation and a 27% decrease of cortical thicknesses (Fig. 5).

Normal bone structure was found in the distal femoral epiphysis of control animals (Fig. 6a).

The trabeculae of the distal femoral epiphysis of ML-treated animals were thin, short and rare. The cell layer zones were well distinguishable. The

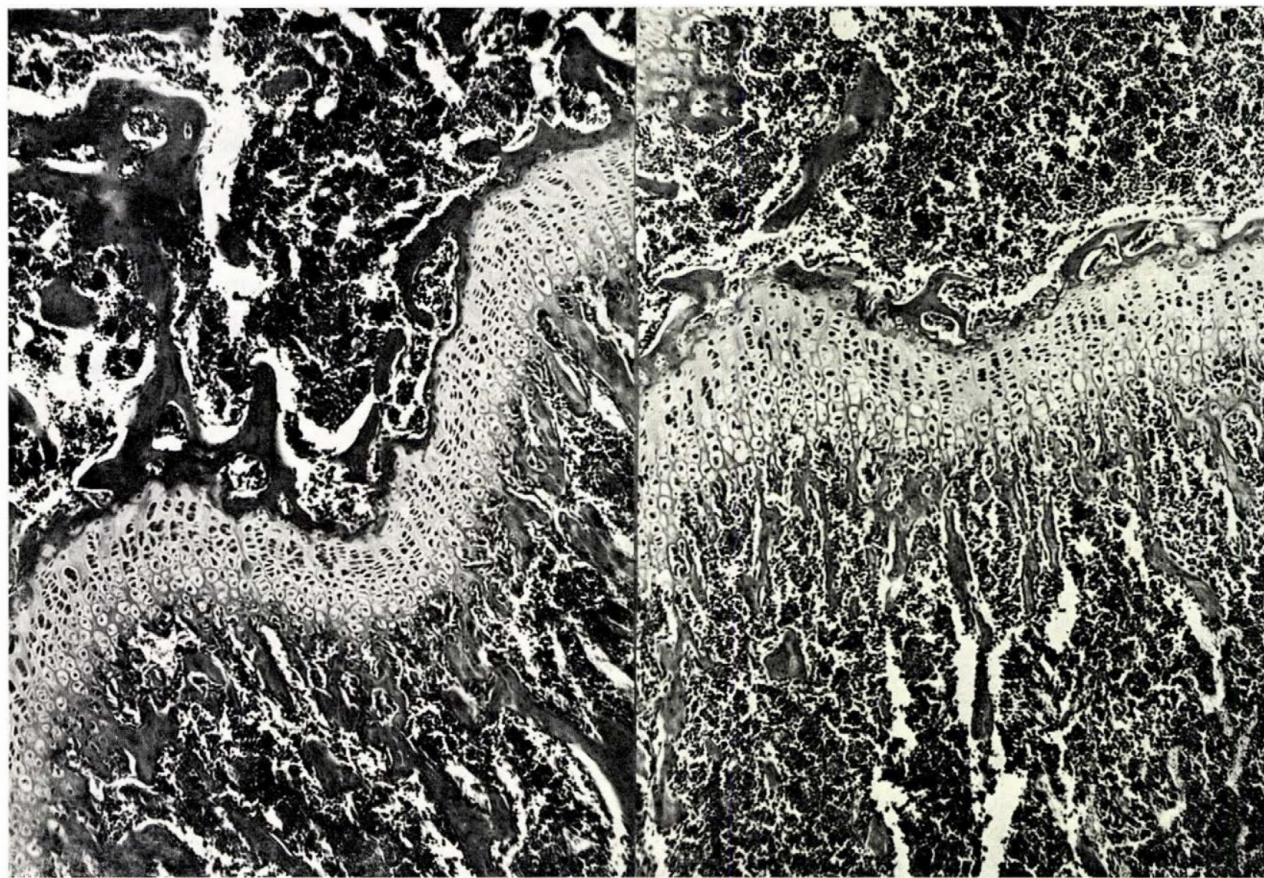


Fig. 3. Distal femoral growth plate of control (a) and ATS-treated (b) mice; haematoxylin—eosin ($\times 60$)

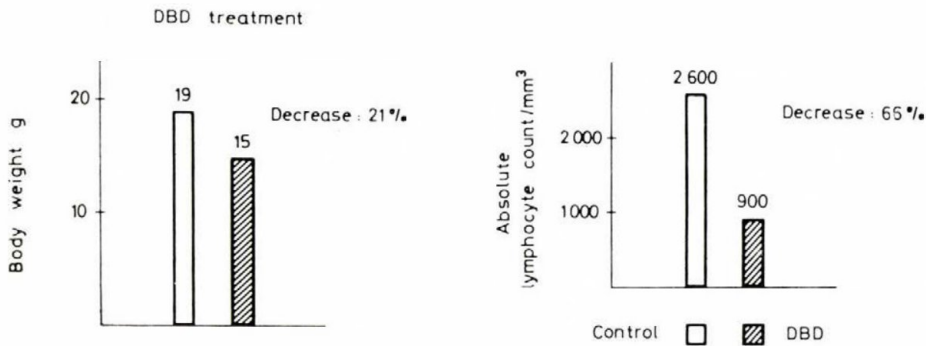


Fig. 4. Mean body weight and absolute lymphocyte count of ML-treated and control mice

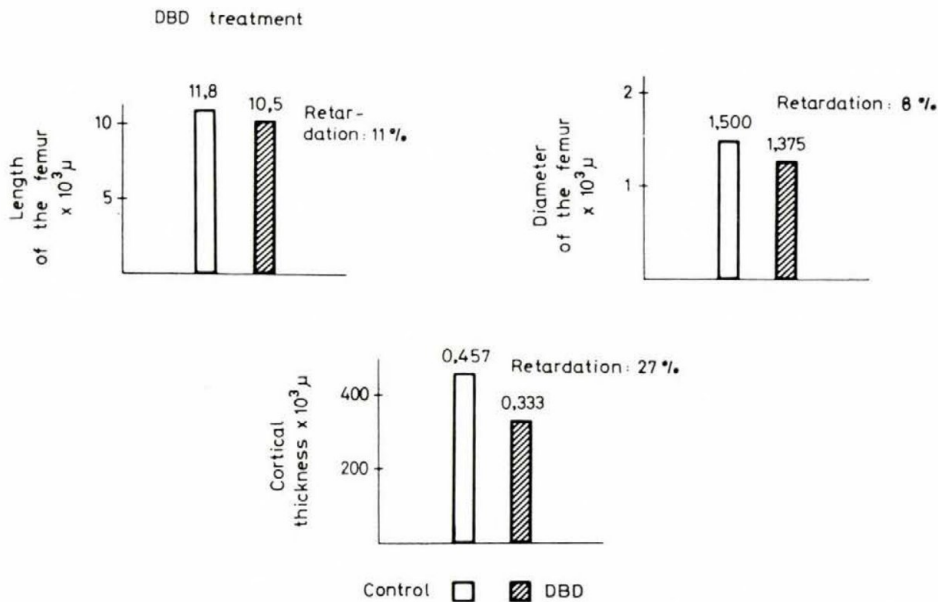


Fig. 5. Mean radiomicrometric measurements of ML-treated and control mice

primary spongy bone trabeculae were thinner and their number was significantly lower in ML-treated animals than in the controls. Osteoblastic activity showed a moderate decrease on their edges. The diaphyseal cortices were thinner (Fig. 6b).

Alcianophilia was found in the intercellular substance of growth plates of control mice and PAS positivity in that of ML-treated animals, indicating an alteration of matrix formation.

Table I summarizes the results of the studies.

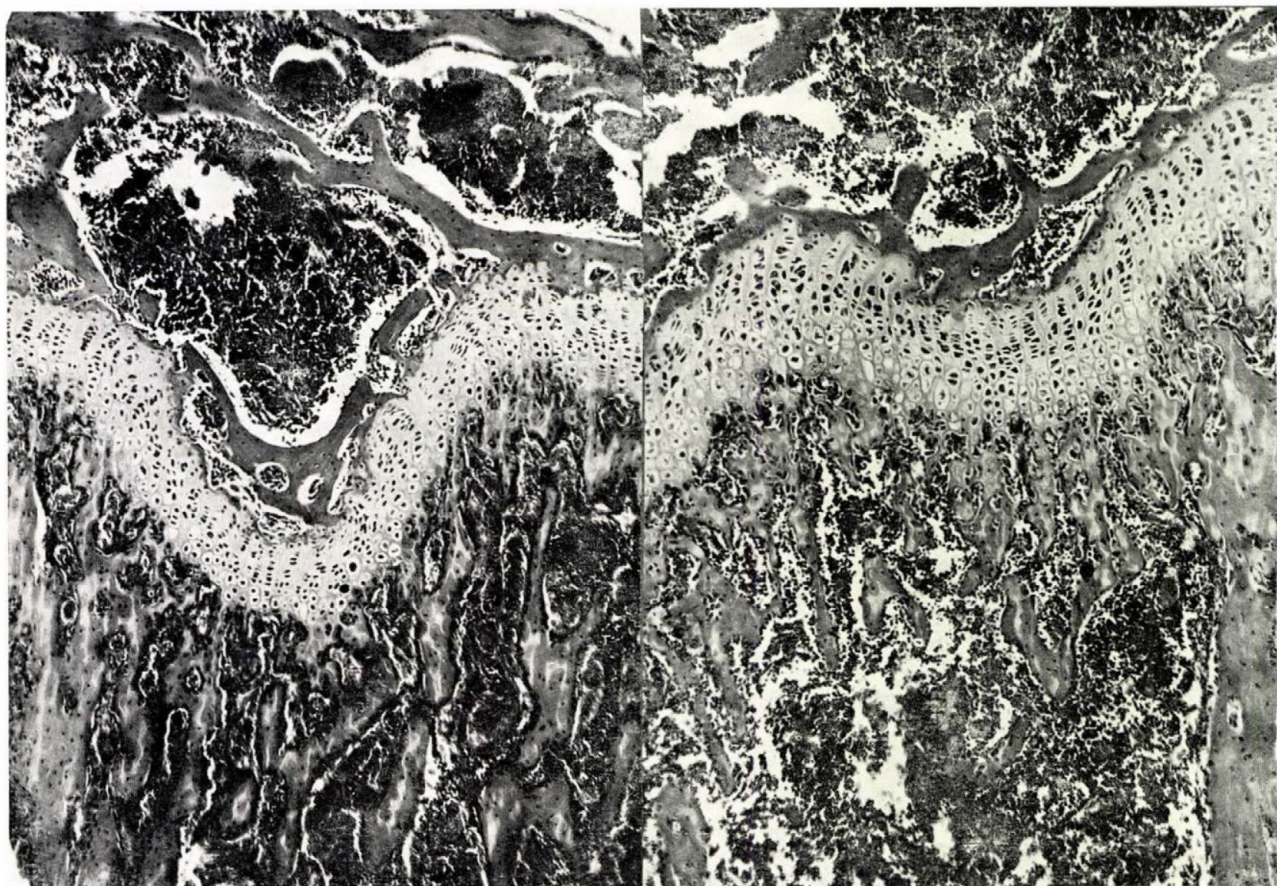


Fig. 6. Distal femoral growth plate of control (a) and ML-treated (b) mice; haematoxylin-eosin ($\times 60$)

Table I
Summary of the results

Group of animals	Femur		
	longitudinal retardation, per cent	diametric retardation, per cent	retardation of cortical thicknesses, per cent
Neonatally thymectomized 3-week-old mice	20	16	37
Neonatally thymectomized 6-week-old mice	22	16	45
Germ-free	14	10	42
GVH	16	11	41
ATS	8	8	28
ML	11	8	27

Discussion

The results indicated that both ATS and ML treatment caused disturbances in the bone development of young mice, which manifested itself in a retardation of enchondral ossification and a decrease of femoral cortical thickness. These bone changes were similar to those detected earlier in young mice with their lymphoid system affected by GVH reaction or after neonatal thymectomy.

ATS treatment is known to impair the thymus dependent lymphocytes selectively, resulting thus in an immunosuppressive effect [10].

Both ML treatment and the GVH reaction affect the thymus dependent lymphoid system and reduce its activity as shown by the reduction of the number of lymphocytes in the blood and lymphoid organs as well as by a decrease of the cellular immune response [11—15].

Though ATS and ML treatment as well as a GVH reaction may impair not only the thymus dependent lymphocytes but also other cells and functions, the changes observed by us in the skeletal system of animals with affected lymphoid system, seem to support the mentioned theory [4]. The fact that a disturbance of skeletal growth was found to be associated in each of the states with a lack of thymus dependent lymphocytes or with their impairment, indicates that the bone changes had a similar background.

According to radiomicrometric measurements, the femoral growth retardation was slighter in ATS- and ML-treated animals than in germ-free mice or in mice subjected to neonatal thymectomy or suffering from a GVH reaction.

Thus, the severity of bone changes was always in direct correlation with the degree of disturbance of the thymus dependent lymphoid system.

Our results allowed the conclusion that the intactness and normal function of the thymus and thymus dependent lymphoid systems are not only requirements of immunological adaptation but also of normal bone development.

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CHEMICAL ACTION OF INFLUENZA VIRUS ON THE RED BLOOD CELL MEMBRANE

I. ACTION ON MEMBRANE NEURAMINIC ACIDS

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(Received November 1, 1973)

Summary. The chemical action of influenza virus strains PR8 and Lee and of periodate on the neuraminic acid containing lipids of human red blood cells has been investigated by thin layer chromatography. The results showed that haemagglutination by the two strains as well as periodate treatment induced manifold and profound chemical alterations in the neuraminic acid-containing lipids. The changes produced by strains PR8 and Lee differed from each other and from those caused by periodate. Alterations in the neuraminic acid-containing lipids induced by heated strain PR8 were identical with those obtained after haemagglutination by the native virus, indicating that virus neuraminidase is not inactivated by heat. Thus, differences in the haemagglutinating ability between native and heated virus are not due to enzyme inactivation.

Since the fundamental works of HIRST [1] and BURNET *et al.* [2, 3] new data of considerable interest have been published on the chemical changes produced on the red blood cell-receptor in influenza virus haemagglutination [4–11] and on the functioning of the viral enzyme [12–14]. In view of the new findings we have decided to investigate these chemical changes by chemical methods directly on the red blood cell.

Materials and methods

In all experiments freshly drawn and citrate-washed human RBC were used. For haemagglutination the freshly harvested allantoic fluid of embryonated eggs infected with influenza strain PR8 or Lee maintained in this Institute was employed. Periodate pretreatment of RBC was performed by suspending 10 ml of an 1% RBC-suspension in 50 ml 0.002 M KIO_4 in saline for 10 min. The pretreatment was stopped by a tenfold dilution of the suspension with saline, followed by centrifugation and washing with saline three times. For heat treatment, the freshly harvested allantoic fluid was placed in a 56°C water-bath for 30 min. The efficiency of heat treatment was checked by a separate test.

In order to minimize the possibility of erroneous conclusions, in every experiment care was taken to assure the strict comparability of the results; for testing native and haemagglutinated RBC portions of one and the same blood sample were used and the whole experiment, *i.e.* processing of RBC and chemical analysis were performed on the same day. The same procedure was followed when comparing the action on RBC of native and heated virus.

In preliminary experiments it was established that uninfected allantoic fluid did not affect chemically the investigated compounds in RBC, just as infected allantoic fluid, after removal of the virus by adsorption to BaSO_4 [15], did not alter these components.

Lipids were extracted from RBC by using a slightly modified form [16] of the method by FOLCH *et al.* [17]. To improve extraction, the solvent was added to the freshly prepared aqueous haemolysate under stirring with a magnetic stirrer for 30 min. The filtered RBC fragments were homogenized for 5–10 min in the extracting solvent with the aid of a MSE homogenizer and this procedure was repeated four times with fresh solvent portions. After

deproteinization with 0.2 vol 0.05 M KCl solution, the pooled filtrates were evaporated in N_2 -atmosphere under reduced pressure. The residue was dissolved in chloroform and the amount equivalent to 50–200 μ g of dry material was used for thin layer chromatography (TLC).

The neuraminic acids in the extract were studied by a two-dimensional TLC-method published earlier [18].

This procedure permitted a tentative qualitative screening of the composition of complex biological preparations; however, for the exact chemical identification and quantitative assessment of the compounds corresponding to the spots, other methods will have to be employed. Since at the present stage of investigations important qualitative differences have been found, it was felt that a report on the most remarkable phenomena would be of interest.

Results

In the chromatograms of the native and haemagglutinated RBC appreciable differences have been noted.

In the chromatogram of the Folch-extract of native RBC, 7 well-separated spots giving the neuraminic acid reaction have been detected [18]. After haemagglutination by strain PR8, their number increased to 9. Some of them differed from the corresponding spots given by native RBC not only in R_F value but also in the intensity of the colour, the latter being markedly diminished. Thus, the amount of neuraminic acid was seemingly less in these spots than in the corresponding spots given by native RBC (Fig. 1).

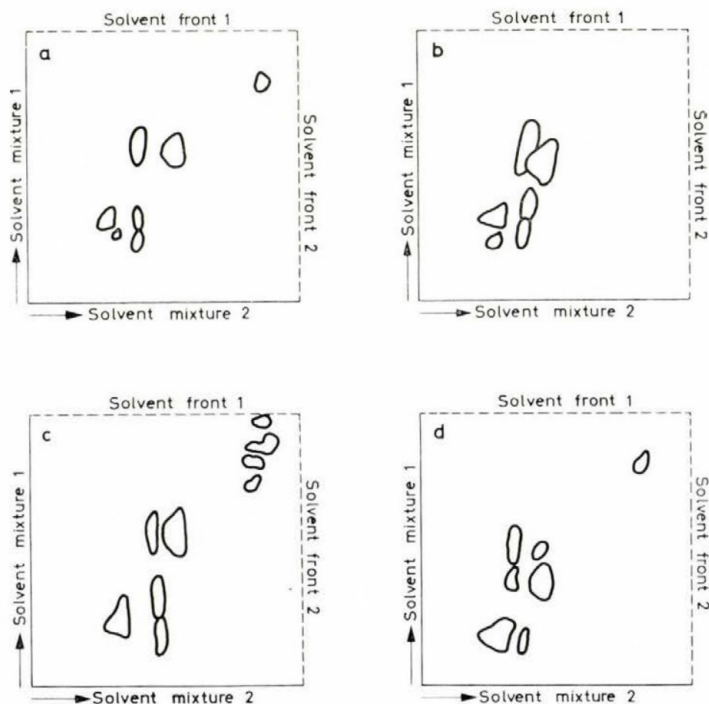


Fig. 1. Two-dimensional TLC chromatograms of the neuraminic acid containing lipids of red blood cells. (a) native RBC; (b) RBC treated with periodate; (c) RBC agglutinated by strain PR8; (d) RBC agglutinated by strain Lee

The number of neuraminic acid spots was increased also after haemagglutination by strain Lee and the change in colour intensity was similar to that induced by strain PR8. The R_F -values differed from those obtained with native RBC and RBC agglutinated by strain PR8 (Fig. 1).

In the extracts of erythrocytes treated with periodate, only 6 spots appeared. Their R_F -values differed from those of native RBC and of the RBC haemagglutinated by the two influenza strains. The decreased intensity of the spots was particularly notable (Fig. 1).

The neuraminic acid spots corresponding to RBC agglutinated by strain PR8 heated to 56°C were identical with those of the RBC agglutinated by the unheated infected allantoic fluid.

Discussion

1. The present experiments have confirmed the results of earlier serological investigations carried out on inhibitors: as a consequence of viral haemagglutination, substantial qualitative and quantitative chemical changes occurred in the neuraminic acids of RBC.

2. In the lipid extract of human RBC, several neuraminic acid-containing lipids were found. The results suggested that in the Hirst-reaction these lipids undergo certain strain specific changes. There was, however, no evidence of a complete destruction of any of these lipids in the course of haemagglutination. Similar findings have been described by KATHAN *et al.* [10] and, in connection with proteolytic enzyme action on RBC mucopeptides, by COOK *et al.* [19].

3. Profound changes in the neuraminic acids of RBC were noted after oxidation with periodate.

4. The reversibility of the agglutination, the "elution" was independent of the neuraminidase-receptor system. Our results indicate that the virion enzyme heated to 56°C is not inactivated. Therefore, heating appears to affect some other active part of the virion, presumably the haemagglutinin, which is somehow altered. Comparing this fact with the findings of ECKERT [13], it may be assumed that the elution observed in the Hirst-reaction is a property attached to the haemagglutinin of the virion, appearing only if the haemagglutinin is intact. In this connection the paper of FAZEKAS DE ST. GROTH and GRAHAM [20] should also be mentioned.

5. Our experiments showed that following haemagglutination by the influenza virus strains PR8 or Lee, different changes occurred in the neuraminic acids of the RBC. This should be considered in the light of findings according to which influenza virus strains differ in their physico-chemical properties, substrate specificity and antigenic properties of their neuraminidases [20–22]. In addition, the action of neuraminidases is affected by the

amount of the neuraminic acid on the surface of the cell and the functioning of the enzyme in each strain is bound to different optimum concentrations of the acid; similarly, the haemagglutinins in various strains also differ from each other, as demonstrated by differences in the antigenic properties [23-29].

The experimentally confirmed substrate specificity of virion neuraminidase as well as the changes caused by virus haemagglutination in the neuraminic acids, may explain the inagglutinability of RBC acquired in the course of the Hirst-reaction. It is evident that neuraminic acids and lipids considerably altered in their chemical properties cannot serve as substrates for the neuraminidases contained in native RBC. They may, however, serve as substrates for neuraminidases and haemagglutinins of another substrate specificity. In such a case previously agglutinated and subsequently eluted RBC may become reagglutinated by another strain.

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CHEMICAL ACTION OF INFLUENZA VIRUS ON THE RED BLOOD CELL MEMBRANE

II. ACTION ON MEMBRANE LIPIDS AND AMINO ACIDS

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Summary. Haemagglutination by influenza virus strains PR8 and Lee as well as treatment of red blood cells by periodate produced numerous and manifold changes in the lipids and the amino acid composition of the erythrocytes. The changes induced by the influenza strains differed from each other and from those induced by periodate. Alterations in the membrane lipids observed after haemagglutination by heat treated strain PR8 and by its native form were identical.

Materials and methods

The materials used and the processing of red blood cells (RBC) have been described in Part I of this study.

In the investigation of lipids a two-dimensional TLC-method [1] and the unidimensional TLC-method proposed by GRANZER [2] were applied. In the two-dimensional method the following colour-developing reagents were used: phosphomolybdic acid [3], iodine [4], ammonium molybdate [5], ninhydrin [6], Dragendorff's reagent, diphenylamine [7], rhodamine-B [8] and Nile-blue [9]. In the unidimensional TLC only phosphomolybdic acid was employed.

The chloroform-methanol phase of RBC extracts was tested separately for amino acids. Half of the organic phase was evaporated at room temperature under reduced pressure in N_2 -atmosphere. The residue was taken up in 0.5 ml of distilled water and after deproteinization by acetone containing 1% concentrated HCl, the suspension was allowed to stand for a day.

The precipitate was centrifuged and the supernatant evaporated. The dry residue was taken up in 0.1 ml of distilled water and delipidized by shaking with 3 ml of ether. The aqueous layer was evaporated to dryness and taken up in 0.05 ml of 0.01 N HCl. The total solution was used for TLC.

The aqueous phase of RBC extracts was evaporated as described above and the dry residue was dissolved in 0.5 ml of 0.01 N HCl containing 1% propanol; 0.005 ml of this solution was used for TLC. A similar procedure was followed to investigate the amino acid composition in the infected allantoic fluid used for haemagglutination in the supernatant obtained after haemagglutination, in the eluate of RBC and finally in the uninfected allantoic fluid.

For TLC, a 250 μ layer of cellulose powder MN 300 was prepared. Development was carried out according to the two-dimensional method of VON ARX and NEHER [10], using three types of solvent systems and the two-dimensional method of WHITE [11]. A standard solution containing 23 amino acids present in the human organism was run simultaneously on each plate. Detection was carried out by spraying with the polychromatic ninhydrin- $Cu(NO_3)_2$ -collidine reagent [12]. The individual amino acids were tentatively identified by the colour and R_F value of the spots and by comparison with standard amino acids.

Results

1. In the Folch-extract of the native RBC, 38 substances were detected differing in R_F values and colour of the spots. Three of the spots displaying low R_F values and one appearing in a higher region of the chromatogram gave

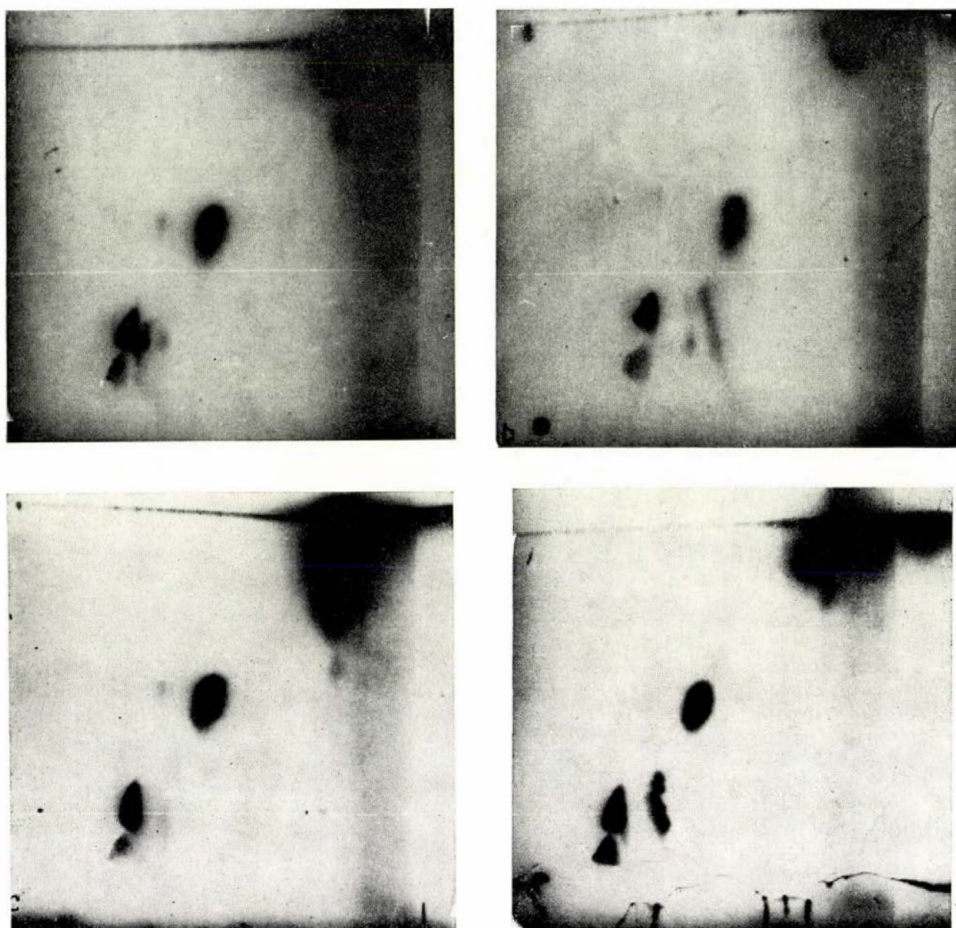


Fig. 1. Two-dimensional TLC chromatograms of Folch-extracts of RBC. Detection reagent: phosphomolybdic acid. (A) native RBC; (B) RBC treated with periodate; (C) RBC agglutinated by strain PR8; (D) RBC agglutinated by strain Lee

none except the ninhydrin reaction representing presumably amino acids. The remaining 34 spots gave some of the reactions characteristic of lipids, 9 of them also reacted with ninhydrin, 13 with Dragendorff's reagent, and among the latter three gave the ninhydrin as well as the Dragendorff-reaction; 11 lipids reacted with Nile-blue. After haemagglutination with strain PR8, changes were noted in the R_F value of all the spots obtained by two-dimensional TLC and in the colour of numerous spots. The number of spots giving the ninhydrin reaction rose to 12, the number of those reacting with Dragendorff's reagent decreased by three, the number of those giving both reactions rose by one, finally, the number of spots giving the Nile-blue reaction decreased by three (Fig. 1).

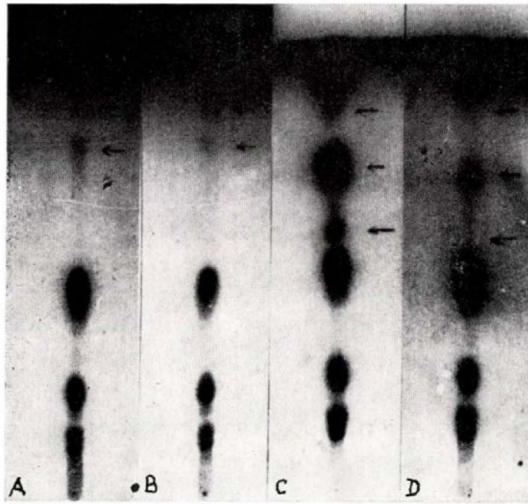


Fig. 2. Unidimensional TLC chromatograms of Folch-extracts of RBC. Detection reagent: phosphomolybdic acid. (A) native RBC; (B) RBC treated with periodate; (C) RBC agglutinated by strain PR8; (D) RBC agglutinated by strain Lee

In the unidimensional system, the colour intensity in spots corresponding to free fatty acids and phosphatidic acids increased considerably in comparison with native RBC (Fig. 2).

The two-dimensional TLC of RBC agglutinated by strain Lee yielded results differing as to the lipid composition of both native RBC and RBC agglutinated by strain PR8 (Fig. 1). With the unidimensional method an increase in the quantity of free fatty acids and phosphatidic acids was noted as compared with native RBC. This increase, however, was less than in the case of strain PR8 (Fig. 2).

Following periodate treatment of the RBC, two-dimensional TLC revealed marked differences in the lipid composition of the native RBC and of those agglutinated with the influenza-strains (Fig. 1). In the unidimensional test, however, no increase of free fatty acids and phosphatidic acids was observed (Fig. 1).

After haemagglutination with heated PR8 strains neither the two-dimensional nor the unidimensional TLC revealed differences in the lipids of RBC extracts as compared with those of RBC agglutinated by native virus.

2. In the chloroform, methanol and aqueous phase of native RBC the following amino acids were detected: cysteine, cystine, asparagine, glycine, lysine, arginine, aspartic acid, glutamic acid, glutamine, β -alanine, tyrosine, serine, histidine, methionine, phenylalanine, α -alanine, leucine, isoleucine, proline, threonine, tryptophan and valine. In addition to the amino acids

reported earlier [13] in this investigation the presence of cysteine, asparagine, aspartic acid and β -alanine has been established. After haemagglutination by strain PR8, threonine and β -alanine were absent and lysine, arginine, tyrosine, methionine and phenylalanine considerably decreased in amount. At the same time hydroxyproline, which was lacking in native RBC, appeared and the amounts of cystine, cysteine, asparagine, aspartic acid, glycine, glutamine, glutaminic acid, α -alanine, serine and histidine increased. Visual comparison of the amino acid composition of the infected allantoic fluid, the native and agglutinated RBC extracts, the supernatant of agglutinated RBC and the eluate revealed that (i) in the infected allantoic fluid, proline and hydroxyproline were absent, whereas they were present in the RBC extracts and in the supernatant of agglutinated RBC; apparently these amino acids were split off during haemagglutination and passed into the supernatant; (ii) asparagine was absent in the supernatant of agglutinated RBC and the infected allantoic fluid, while its amount in the extract of agglutinated RBC surpassed that in native RBC.

Discussion

1. The behaviour in the Hirst-reaction of RBC pretreated with periodate cannot serve as a basis for assuming that the receptor is a carbohydrate. Alpha-glycol groups are found not only in carbohydrates, but also in the glycerol component of lipids and in the carbohydrate components of glycolipids, among others. Primary amino alcoholic groups are also oxidized by periodate at a rapid rate. Thus, for the sensitive histochemical detection of lipids, the Schiff-reaction based on the oxidizing action of periodate can conveniently be applied [14].

2. As to the amino acid composition of native RBC and allantoic fluid, our findings suggest that in the native RBC asparagine is present partially in a masked state. In the course of haemagglutination, the masked part is split off while the residual part thus liberated remains bound to the erythrocyte structure. In connection with the disappearance of threonine from the RBC extracts after haemagglutination, it should be mentioned that according to HOWE *et al.* [15] in virus receptor obtained from RBC among the amino acids threonine and serine were present in highest amounts. In the opinion of ANDERSON *et al.* [16] these two hydroxy-amino acids linking the protein and carbohydrate moieties in glycoproteins are destroyed in the course of alkaline hydrolysis. The disappearance of threonine from RBC extracts as a consequence of viral haemagglutination may be the result of a similar process. Since this effect was demonstrated in RBC extracts obtained with lipid solvents and in view of the numerous experimentally confirmed alterations in the amino acids after viral haemagglutination, it appears probable that the receptor, *i.e.*

the cell surface component linking the virion to the surface of the RBC, is a glycolipoprotein as assumed by HOWE *et al.* [15].

3. Manifold chemical changes occur in the RBC after contact with the virion involving amino acids lipids in addition to neuraminic acids. It is therefore highly improbable that the complex phenomena in the Hirst-reaction should be due to structural changes in a single substance, the receptor.

4. In our experiments several changes were recorded in the lipid extract of RBC, particularly with ninhydrin and Dragendorff's reagent which denote polar lipid groups. It has been reported that the electric charges on RBC membranes are determined almost exclusively by the charge of neuraminic acid on the surface of the membrane [17]. Cell membranes lose their electric charges in interaction with virions [18] neuraminidase [19] and trypsin [17]. Thus, as a result of the neuraminidase action of the influenza virion, neuraminic acid, *i.e.* the constituent maintaining the electrostatic equilibrium of the cell membrane, is split off from the receptor molecule and changes in the chemical composition and physico-chemical properties (*e.g.* swelling and porosity) of the membrane are likely to occur. Especially changes in the swelling properties may increase the agglutinability of RBC [20].

5. The study of the chemical changes in the Hirst-reaction allows to investigate the action of a virus on easily accessible cell membranes. The knowledge thus obtained will greatly contribute to the understanding of viral action on susceptible cells, as membranes on the surface of the cells are similar in structure to those of RBC. Similar membranes are present in the cell interior, playing a significant role in the intracellular development of the virion and in the synthesis of proteins in general. Accordingly, the cytopathogenic effect of a virion may be considered a result of the interaction between virion and lipoprotein membrane of the lysosome [21–23]. Morphological studies showed that during maturation lipid containing viruses (*e.g.* myxoviruses) are closely attached to lipoproteins of the nuclear membranes and the endoplasmatic reticulum [24]. On the other hand, BLOUGH *et al.* [25–28] have shown that lipids as well as vitamin A affecting the surface conditions of the membrane deeply influence the morphological and biological characteristics of the developing virus.

DE BURGH *et al.* [29] reported that influenza virus agglutinates mitochondria isolated from liver cells of rats and mice. After pretreatment with RDE, this agglutinability is however lost. The observation of MARCUS *et al.* [30] that influenza virus splits neuraminic acid from isolated nuclear membranes is in agreement with this finding. It was found that the lipid composition of influenza virus lipids derived from the host cell corresponds mainly to that of the nuclear membrane [31, 32]. Penetration, intracellular cycle and variability of the virus are presumably functions of the interaction between virion and cell membrane.

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THE EFFECT OF SUPRAOPTIMAL TEMPERATURE ON THE MULTIPLICATION OF DIFFERENT CYTOMEGALOVIRUS STRAINS

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Summary. The virion synthesis by five human cytomegalovirus (CMV) strains in human embryonic fibroblast cultures was stopped by incubation of the infected cultures at 40°C. At this temperature the antigens appeared diffusely filling the nucleus and the cytoplasm. The blocking effect of the elevated temperature was exerted in the same period of the reproduction cycle as the inhibitory effect of cytosine arabinoside (ara-C). In cell cultures infected with CMV and incubated first at 40°C, then at 37°C, the synthesis of infectious virus started again, thus the abortive cycle developed at 40°C was reversible. The inhibition of virus multiplication cannot be attributed to the thermosensitive events in the normal function of the host cell.

At 40°C the virus yield of cultures infected by herpes simplex virus type 2 (HSV-2) is low or there is practically no virion synthesis, whereas the multiplication HSV type 1 (HSV-1) strains is scarcely suppressed [1–3].

Recently it has been shown that cell cultures infected with HSV-2, viz., hamster embryonic fibroblasts [4], human embryonic lung cells [5] and chicken embryonic fibroblasts [6], when incubated at 40°C or 42°C, showed changes characteristic of transformation.

In the present work the multiplication of human cytomegalovirus (CMV) strains was investigated at 40°C. It seemed to be of interest to study whether the viral cytopathogenic effect (CPE) and infectious CMV synthesis were inhibited and whether there are any strain differences in replication at that temperature. Four of the 5 CMV strains under study displayed serological strain differences in earlier studies [7, 8].

Materials and methods

Cell cultures. Primary or secondary human embryonic cells were cultured and maintained in Parker's 199 enriched by 10% calf serum.

Virus strains. Strain CMV-Rawles was received from Dr. STERN (St. George's Hospital Virus Laboratory, London). The strain has been maintained in this laboratory since 1964, and characterized by us [9]. Strains CMV-Ad-169, Davis, C-87 and T-27 were received from Dr. H. K. ANDERSEN (Institute of Medical Microbiology, University of Aarhus, Denmark) and described elsewhere [7]. Strains HSV-1 HIL and HSV-2 LOV were received from Professor P. WILDY (Birmingham, England) and characterized earlier [6].

Effect of incubation at 40°C on the multiplication of CMV and HSV strains. Tube cultures of human embryonic fibroblasts were infected with CMV at a multiplicity of infection 1 TCID₅₀/cell. After a one-hour adsorption period at 37°C half of the cultures were incubated at 37°C, the

other half at 40°C. Infective virus was titrated 1 hr after inoculation and on days 1–6 after infection. With HSV-1 and HSV-2 the multiplicity of infection was 0.1 TCID₅₀/cell and the infective virus was titrated 1, 4, 8, 12, 16, 24 and 32 hr after infection. Otherwise the procedures were the same as with the CMV strains.

Inactivation of intra and extracellular CMV at 40°C. Cultures infected at a multiplicity of 1 TCID₅₀/cell were incubated at 37°C. When the diffuse CPE had appeared and the virus yield had reached its maximum on the third day the cultures were transferred to 40°C. Total infectivity was determined 24 or 48 hr thereafter.

Recovery of CMV after incubation at 40°C. Cultures were inoculated and virus was adsorbed as described above and incubated at 40°C for various periods of time, then transferred to 37°C and the virus yields were measured.

Determination of virus yield. Cell cultures infected with CMV or HSV were suspended in the original medium by digestion with trypsin at times indicated under Results and disintegrated by ultrasonication. The sonicate was centrifuged at low speed and the supernatant was titrated in tube cultures of human embryonic fibroblasts. The virus titre was calculated by the REED–MUENCH formula [10].

Determination of CMV-specific antigens. Cover-slip cultures were fixed and stained as described elsewhere [11]. As antiserum, a 1/10 dilution of a pool of 10 convalescent sera was used.

Treatment with ara-C and transfer of cultures to 40°C. Cell cultures were infected with 1 TCID₅₀/cell of the Rawles strain. After an adsorption period of 1 hr the cultures were incubated at 37°C. From parallel cultures tubes were transferred to 40°C 12, 24 and 36 hr after infection or treated with 15 µg/ml ara-C. The infectious virus yield was determined in the 48th hr.

Effect of incubation at 40°C on the multiplication of embryonic fibroblast cells. Primary cell cultures were trypsinized, and secondary cultures were started with an equal cell number at 40°C and 37°C. The cell count was followed up for 5 days.

Effect of pre-incubation at 40°C on virus yield. Human embryonic fibroblast cultures were preincubated for 5 days at 40°C, then infected with 1 TCID₅₀/cell of the Rawles strain. After an adsorption period at 37°C for 1 hr the tubes were postincubated at 40°C. Control cultures were pre and postincubated at 37°C. Infectious virus yield was followed up for 3 days.

Results

Effect of incubation at 40°C on CPE, on virus synthesis and on the development of viral antigens in cells infected with various CMV strains. *Effect of incubation at 40°C on the multiplication of HSV-1 and HSV-2.* Fig. 1 shows that no infectious virus could be demonstrated in the cell cultures incubated at 40°C following inoculation of the cultures with either of the CMV strains. The cell cultures incubated at 37°C yielded regular multiplication curves. At 40°C, CPE developed in 24–48 hr, but the cell cultures regenerated subsequently.

Multiplication of HSV strains at 40°C remained below the control value. The difference was 1 log unit for HSV-1 and 4 log units for HSV-2.

Of the intracellular CMV antigens, that diffusely filling the nucleus and that diffusely localized to the cytoplasm appeared 24–48 hr after inoculation, and remained demonstrable in 90% of the cells during the whole period of incubation. Further antigenic components characteristic of CMV [12] failed to develop (Fig. 2). There were no considerable strain differences in these respects.

It was supposed that some of the strains synthesized complete virus at 40°C and it was the rapid inactivation of the progeny virus that prevented the infectious virus from being detected. For this reason inactivation at 40°C was investigated.

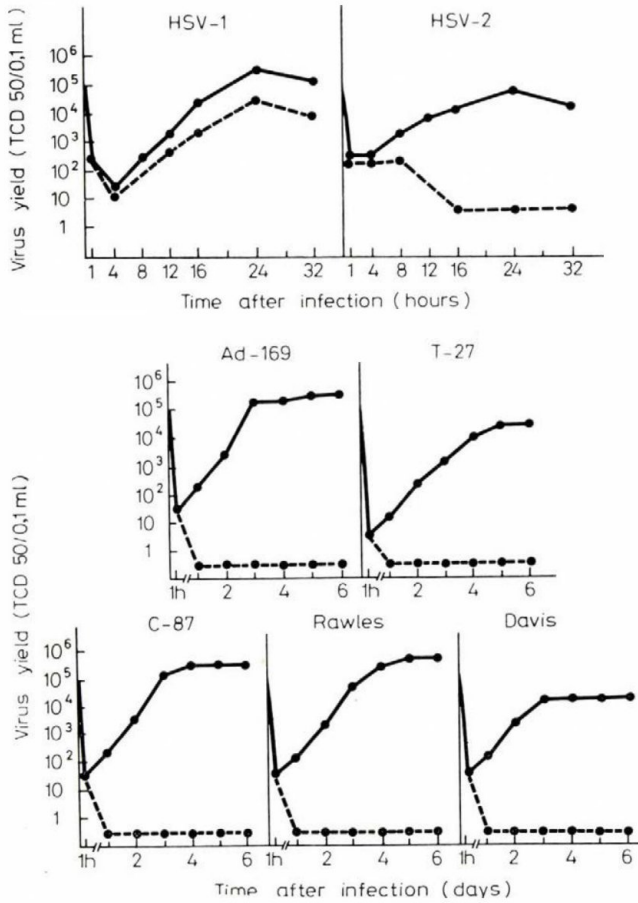


Fig. 1. Effect of incubation at 40°C on the production of infectious virus. Virus strains: Ad-169, T-27, Davies, C-87 and Rawles strains of CMV, a HSV-1 and a HSV-2 strain. ●—● Cultures incubated at 37°C. ●---● Cultures incubated at 40°C

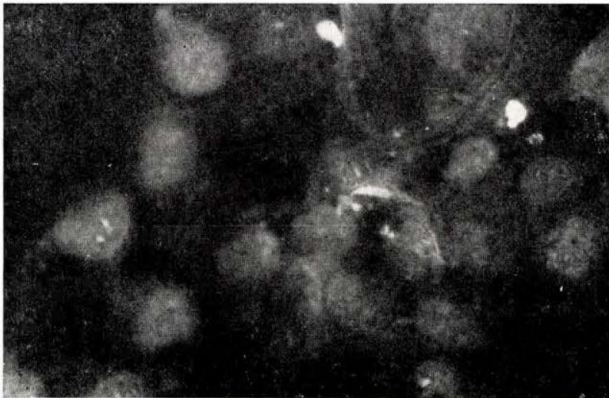


Fig. 2. Antigens filling up the cytoplasm and the nucleus in cells incubated at 40°C

Inactivation of infectious virus at 40°C. Transfer to 40°C of cultures showing diffuse CPE resulted in a reduction of the virus yield by 0.5–1 log unit in the first 24 hr and by 1.5–2 log units in the first 48 hr after transfer. There were no appreciable strain differences (Fig. 3). Accordingly, thermal inactivation, though it existed, failed to explain the disappearance or the great reduction of the infectious virus in the above-described experiments.

Recovery of infectious virus from cultures transferred from 40°C to 37°C. Infected cultures incubated at 40°C were transferred to 37°C on the 4th, 6th or 9th day after infection. Irrespective of the strain, the virus could be recovered even after 9 days at 40°C and was first demonstrated on the 4th or 5th day after transfer. The amount of recoverable virus did not depend on the length of incubation at 40°C (Fig. 4). Accordingly, an abortive cycle proceeded at 40°C, which then turned into a complete cycle at 37°C.

Comparison of the effect of incubation at 40°C with that of ara-C. In these experiments the Rawles strain was used. Forty-eight hr after infection no virus was detectable in the cultures to which ara-C was added at the time of the infection or 12 hr thereafter, but virus was present in those to which the ara-C was added 24 or 36 hr after the infection. These results indicate that virus DNA synthesis had started in the cultures between 12 and 24 hr after infection. Considering that the virus yield was smaller in the cultures treated in the 24th or 36th hr ($10^{3.5}$ and $10^{3.75}$ TCID₅₀/0.1 ml, respectively) than in the untreated controls ($10^{4.25}$ TCID₅₀/0.1 ml), it was assumed that DNA synthesis continued after the 36th hr [12].

When parallel cultures were transferred to 40°C in the first or 12th hr after infection, no virus could be detected, whereas in those transferred in the 24th or 36th hr the virus yield was smaller ($10^{2.25}$ and $10^{2.5}$ TCID₅₀/0.1 ml, respectively) than in the cultures treated with ara-C at the respective times (Table I).

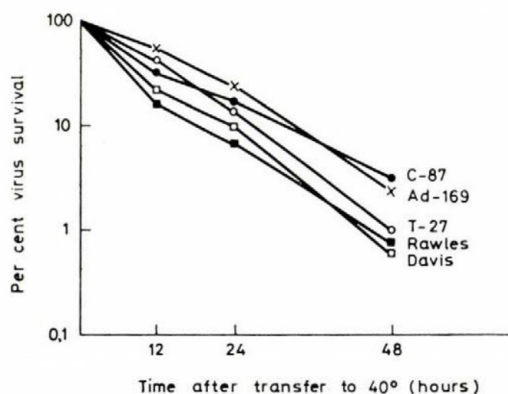


Fig. 3. Inactivation of infectious CMV in cell cultures incubated first at 37°C and subsequently at 40°C

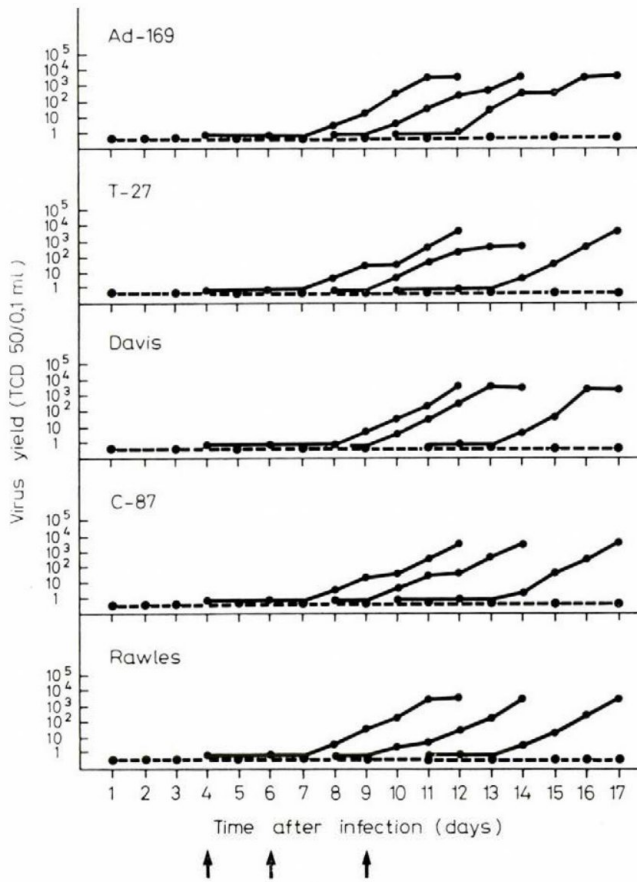


Fig. 4. Recovery of infectious CMV from infected cultures incubated first at 40°C and subsequently at 37°C. Arrows indicate the time of temperature change. ●—● Cultures transferred to 37°C; ●---● Cultures incubated at 40°C throughout

Table I

Effect of 15 μ /ml ara-C and 40°C temperature on infectious virus yield

Time of treatment with ara-C (hr after inoculation)	Virus yield at 48 hr (log TCID ₅₀ /0.1 ml)	Time of transfer of cultures from 37° to 40° (hr after inoculation)	Virus yield at 48 hr (log TCID ₅₀ /0.1 ml)
1	—	1	—
12	—	12	—
24	10 ^{3.5}	24	10 ^{2.25}
36	10 ^{3.75}	36	10 ^{2.5}
—	10 ^{4.25}	—	10 ^{4.25}

Multiplication of human embryonic fibroblasts at 40°C. Table II shows the multiplication of human embryonic cells at 37°C and 40°C. In cultures incubated at 40°C for one or two days immediately after passage the cell count was lower than in the 37°C control cultures. However, from the 3rd day on there was no difference in the cell counts.

Table II

Multiplication of human embryonic fibroblast cells at 37°C and 40°C

Days after	No. of cells at	
	37°C	40°C
0*	80 000	80 000
1	86 000	33 000
2	130 000	86 000
3	146 000	110 000
4	150 000	120 000
5	160 000	140 000

* Day of trypsinization

Infectious virus yield of human fibroblast cells preincubated at 40°C. The virus yield was not reduced by preincubation of the cultures at 40°C for 5 days (Table III).

Table III

Effect of pre-incubation at 40°C for 5 days

Cultures	Virus yield TCID ₅₀ /0.1 ml at 37° on		
	1st day	2nd day	3rd day
Pre-incubated at 40°C	10 ^{2.25}	10 ^{2.75}	10 ^{4.5}
Pre-incubated at 37°C	10 ^{2.25}	10 ^{3.25}	10 ^{4.75}

Discussion

We have failed in demonstrating infectious CMV in infected cell cultures incubated at 40°C. This could not be attributed to a thermal inactivation of the introduced virus, for virus synthesis was resumed even after 9-day incubation at 40°C as the incubation of the cultures was continued at 37°C (Fig. 4). Furthermore, the viral information was, at least partially, expressed at 40°C, as shown by the appearance of virus-specific antigens.

The lack of infectious virus in the cell cultures incubated at 40°C could not be attributed to the thermal inactivation of the newly-synthesized virus,

either, for virus was demonstrable in the cultures in which the virus had been produced at 37°C even after 24 or 48 hr postincubation at 40°C. In fact, in these cultures the virus yield was lower by 1 and 2 log units, respectively, as compared with the control values (Fig. 3). This, however, failed to explain the 5 log unit difference between the virus yields of cultures incubated at 37°C and 40°C.

One might suppose that the virus produced at 40°C is inactivated at 40°C at a higher rate than that produced at 37°C. Fig. 4 shows that in cell cultures transferred from 40°C to 37°C virus was not demonstrable until the 4th or 5th day after the transfer. If this virus had been synthesized at 40°C, it should have appeared on the first day already. Consequently, the failure to demonstrate infectious virus at 40°C was not due to the thermal inactivation of either the introduced or the newly-synthesized virus but the virus synthesis itself was inhibited.

The effect of incubation at 40°C was compared with that of the DNA inhibitor ara-C. Synthesis of infectious virus was completely blocked if ara-C was added, or the cultures were transferred to 40°C, 1 or 12 hr after the infection. Ara-C treatment after the 24th or 36th hr resulted in a smaller reduction in virus yield measured at 48 hr than incubation at 40°C from the same time on. It cannot be excluded that besides DNA synthesis some other process is inhibited at 40°C and thermal inactivation might also play some role.

We have failed to show any difference in behaviour at supraoptimal temperatures among serologically different CMV [7, 8]. Furthermore, the abortive cycle proceeding at 40°C was completed at subsequent incubation at 37°C, irrespective of the strain.

HSV-1 and HSV-2 viruses replicate at different rates at supraoptimal temperatures. In this the species of the cells and their type (epitheloid or fibroblast) may also play a role [3]. In this respect the difference between HSV-1 and HSV-2 viruses is very pronounced in hamster embryonic fibroblasts, whereas it is slight as tested in rabbit kidney cells [2, 3]. On this basis it seems possible that CMV strains could also be differentiated by culturing them at supraoptimal temperatures in other cell cultures. In the human embryonic cell cultures applied in the present experiments, HSV-1 strains multiplied well at, and only HSV-2 strains were considerably inhibited by, elevated temperatures (Fig. 1). It is therefore unlikely that the heat-sensitive processes of the host cells are responsible for the blocking of the reproduction of CMV. This is supported by the fact that the damage due to incubation at 40°C is too slight to stop cell multiplication (Table II). Cells pre-incubated for 5 days at 40°C yielded approximately as much virus as those pre-incubated at 37°C (Table III).

WILDY [4] succeeded in transforming hamster embryonic cell cultures with HSV-2 by inhibiting the lytic functions and the synthesis of infectious

virus by high temperatures. The transformed cells proved to be oncogenic in the hamster. GÉDER *et al.* [6] incubated HSV-2-infected chicken embryonic fibroblasts at 40°C, DARAI and MUNK [5] kept human embryonic lung fibroblasts at 42°C. Both kinds of cells acquired properties characteristic of transformation. Thus, we have drawn the following conclusions. (i) All CMV strains are inhibited in their lytic function and in synthesizing infectious virus at 40°C; (ii) at this temperature an abortive cycle arises which can turn into a complete cycle at 37°C; (iii) it seems possible to induce transformation with CMV in cells cultivated at elevated temperatures.

Acknowledgement. The authors are indebted to Mrs. J. HERPAY for excellent technical assistance.

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COURSE OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS INFECTION IN YOUNG AND AGED MICE*

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Summary. The course and histological picture of intracerebral LCM virus infection was found to differ in mice with their thymus displaying an age-dependent involution and in young adult animals. Signs of lymphocytic choriomeningitis were absent in 75% of the aged animals. The change of the characteristic clinical and histological picture referred to the decreased cellular immune response of aged animals, ascribed to the age-dependent involution of the thymus.

The effect of intracerebral lymphocytic choriomeningitis (LCM) virus infection is known to differ in newborn and adult animals. A symptomless carrier state develops in newborn mice while lymphocytic choriomeningitis of fatal outcome in adult ones.[1] A cellular type immune reaction takes place on the pia mater, the antigenicity of which has changed during the course of virus multiplication, and this represents the basis of the neurological symptoms typical of LCM [2].

The course of intracerebral LCM virus infection in neonatally thymectomized adult mice is similar to the effect of neonatal infection [3–7]. In both conditions, in the deficient cellular immune response, which is the consequence of the underdeveloped thymus-dependent lymphoid system, results in a symptomless carrier state.

Our own and other studies have shown that lymphocytic infiltration fails to occur on the pia mater of mice with a developed lymphoid system, if the cellular immune response had been reduced by impairing their thymus-dependent lymphoid system [1, 8–17]. Hence, the course of LCM infection depends on the cellular immune response, *i.e.* on the condition functioning of the thymus-dependent lymphoid system. In the present work we have studied the question whether the course of LCM infection was influenced by the age-dependent involution of the thymus. The consequences of the infection were observed and compared in young adult mice and in aged animals the thymus of which had reached the state of physiological involution.

* Presented at the 6th Congress of the Hungarian Association of Microbiologists, Budapest 1973.

Materials and methods

Experimental animals. Young and old C3H, C57BL and A inbred mice of both sexes were used.

Lymphocytic choriomeningitis virus. WE strain maintained in serial mouse-passages and stored at -70°C in our laboratory, was applied. Mice were injected intracerebrally with 100 to 300 LD₅₀ of the previously titrated virus.

Study of the lymphoid system. Absolute lymphocyte count was determined in blood samples taken under standardized conditions from the caudal vein of uninfected animals. After sacrifice, the relative weights of thymus, spleen and lymph nodes were determined:

$$\text{relative organ weight} = \frac{\text{organ weight, mg}}{\text{body weight, g}}$$

Histological examinations. Brains of mice from the infected group were fixed in formalin and the sections were stained with haematoxylin-eosin.

Results and discussion

Four mouse groups were used. Origin, age and number of the mice, are presented in Table I.

Table I

Origin, age and number of mice belonging to the experimental groups

Groups	No. of animals	C3H	C57BL	A
I	30	6-10 weeks old		
I + LCM	30			
II	30	8-12	12-16	16-24
II + LCM	70	months old		

Mice of the I + LCM and II + LCM groups were simultaneously infected with LCM virus under identical conditions. Mice of groups I and II were left uninfected. Their observation was continued until the 12th day following challenge. The infected animals developed characteristic neurological symptoms (tremor, convulsions). Fig. 1. demonstrates the time-curve of deaths.

Mice of the I + LCM group succumbed 6 to 8 day following the infection, displaying typical neurological symptoms. The death-curve of II + LCM mice was protracted, deaths occurred between the 3rd and 12th day. Neurological symptoms failed to occur in the animals dying between the 3rd and 6th day, while one third of the animals dying between the 6th and 12th day displayed these symptoms. Animals dying without neurological symptoms displayed atrophy (hunched posture, loss of weight, dermatitis, loss of fur; Figs 2, 3).

The brain of mice in the infected group was subjected to histological studies. Lymphocytic infiltration was observed in each mouse of the I + LCM

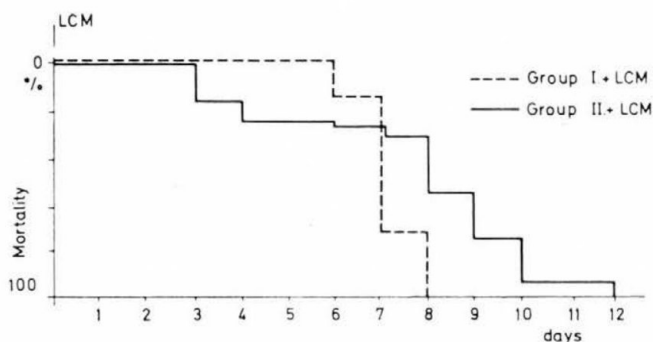


Fig. 1. Time-curve of deaths following intracerebral LCM virus infection



Fig. 2. Aged C3H mouse (group II)

group but only in 25% of the II + LCM group. Characteristic histological changes were lacking in the atrophic animals dying without neurological symptoms. No death occurred in the uninfected groups. Absolute lymphocyte counts and relative lymph organ weights were significantly different in the young and aged mice groups I and II, respectively. These data are presented in Fig. 4.

Absolute lymphocyte count varied between 3800 and 4700, and relative thymus weight between 2.8 and 3.6 g in group I, while in group II the lymphocyte counts varied between 1900 and 2600 and relative thymus weight was less than 1. Seventy-five percent of the aged mice failed to show signs of LCM. The consequences of infection were similar to those observed earlier in mice with their lymphoid system impaired in different ways. Neurological symptoms and a histological picture typical of LCM developed only in a small part of the animals, the remaining ones died later in an atrophic condition without neuro-



Fig. 3. Aged C3H mouse, 11 days after LCM infection (II + LCM group)

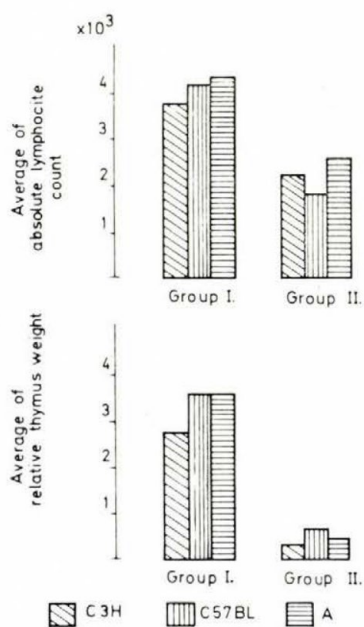


Fig. 4. Mean absolute lymphocyte counts and relative spleen weights in groups I and II

logical symptoms and without lymphocytic infiltration on the pia mater [16-18].

The course of LCM infection in aged mice was indicative of their decreased immune responsiveness. This we ascribed to the age-dependent involution of the thymus.

The present findings refer to the possibility that in old age the course and histological picture of infectious diseases may deviate from their classical appearance and for this the immune responsiveness reduced by the physiological involution of the thymus may be responsible.

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CYTOLOGICAL, CYTOCHEMICAL AND IMMUNO-FLUORESCENCE STUDIES WITH DUGBE VIRUS — A NEW NIGERIAN TICK-BORNE VIRUS*

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Summary. Dugbe virus, a new tick-borne arbovirus from Nigeria, was propagated in continuous porcine kidney (PS) cells, and the cytopathology studied by various staining techniques such as May-Grünwald-Giemsa, methyl-green pyronine, Feulgen, and immuno-fluorescence. The gross cytopathology was slight and infected cells continued to undergo normal mitotic divisions. The outstanding cytopathologic feature was the presence of large basophilic intracytoplasmic inclusion bodies, which were pyroninophilic and Feulgen negative. By immunofluorescence these inclusions were shown to be depots of viral antigen; specific fluorescence was confined to the cytoplasm throughout the replication of the virus.

Dugbe virus is a new tick-borne arbovirus isolated at the Virus Research Laboratory, Ibadan, Nigeria. It is the most common arbovirus in the country; infecting both domestic livestock and man. A sensitive tissue culture system was developed by DAVID-WEST and PORTERFIELD [1], and this has been used to study some of the biological characteristics of the virus. There is need to study the cytopathology in greater detail than was done previously. The studies reported here were therefore designed to achieve this by employing different cytological techniques.

Materials and methods

Virus. The prototype strain of the virus, Ib. Ar. 1792 was used in most of the tests, a second strain, Ib. Ar. 2484 was used in a few tests for comparison.

Tissue culture. A stable line of pig kidney cells, PS cells [2] was used. The growth medium was LEIBOVITZ L15 medium [3] containing 3% fetal calf serum, 10% tryptose phosphate broth, penicillin (100 IU/ml) and streptomycin (100 µg/ml). The cells were incubated at 35°C.

Infection and processing of cultures. Leighton tubes were seeded with PS cell preparation containing 1.5×10^5 cells/ml; these were infected with 5 plaque forming units (p.f.u.) of virus per cell, and incubated at 35°C. At daily intervals up to the 8th day coverslips were removed from infected and control cultures. The culture fluid of the former was titrated for infective virus, while the coverslips were used for various staining procedures as follows.

(a) May-Grünwald-Giemsa stain: the coverslips were washed in saline, and fixed in absolute methanol for 10 min. May-Grünwald stain was applied for 10 min, followed by Giemsa stain (freshly diluted 1 : 15) for 20 min. They were then rinsed in distilled water, destained in two changes of acetone, and cleared, first in acetone-xylol mixture (1 : 1), then in xylol, and finally mounted in balsam.

(b) Methyl-green pyronine staining was done according to TREVAN and SHARROCK [4].

(c) Feulgen staining was made as reported by NEGRONI [5].

* Most of this work was conducted at the National Institute of Medical Research, Mill Hill, London, and supported by a Commonwealth Tropical Medicine Research Fellowship.

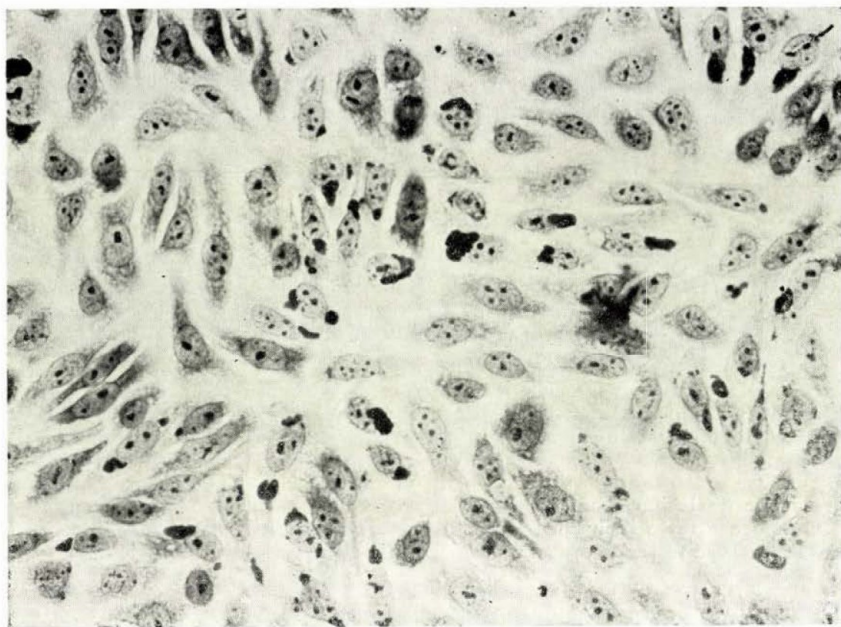


Fig. 1a. Uninfected PS cells (8 days). $\times 400$

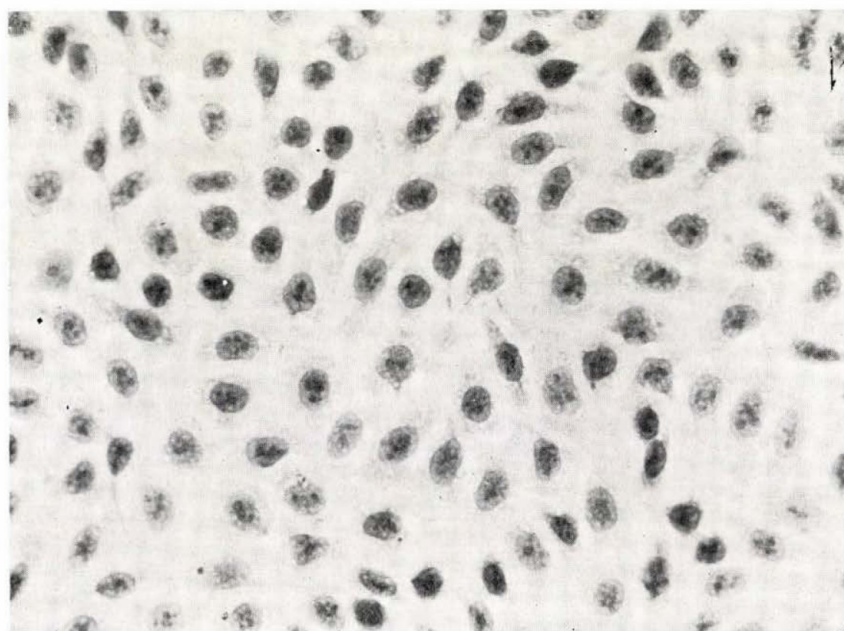


Fig. 1b. PS cells infected with Dugbe virus (8 days); note inclusion bodies. $\times 400$

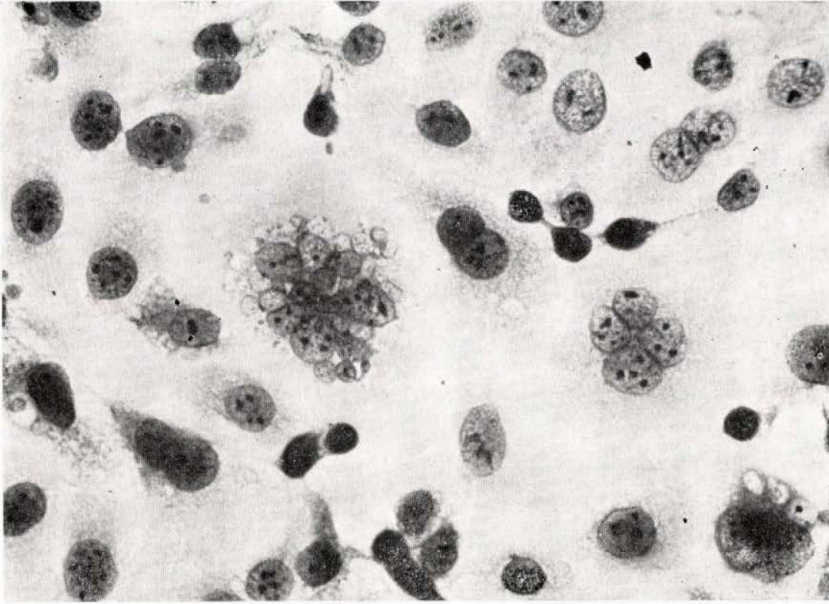


Fig. 1c. Uninfected LLC-MK2 cells (8 days); note multinucleated cells. $\times 400$

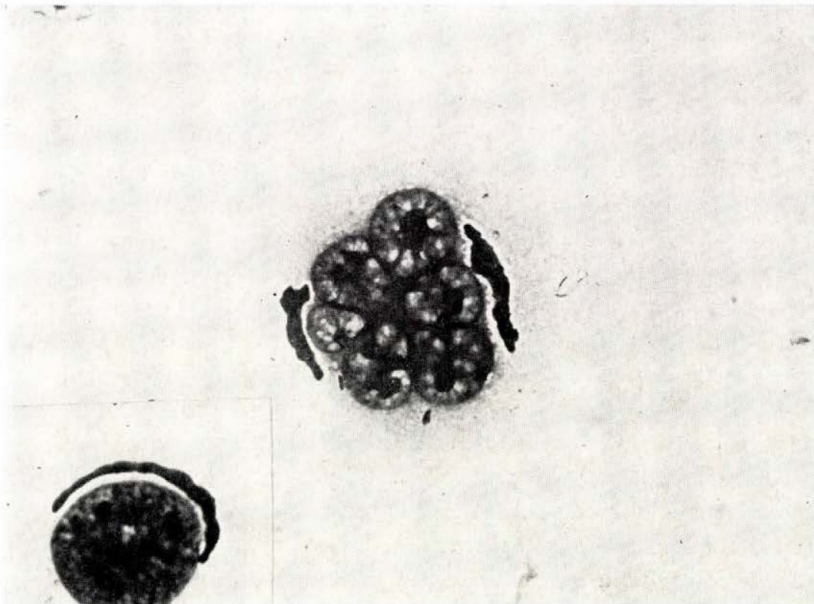


Fig. 1d. LLC-MK2 cells infected with Dugbe virus; note multinucleated cells, and inclusion bodies. $\times 400$. All the above preparations were stained with May-Grünwald-Giemsa

(d) Fluorescent antibody staining. The indirect or sandwich technique was used, with fluorescein isothiocyanate conjugated sheep anti-rabbit globulin. The coverslips were rinsed three times in phosphate buffered saline, pH 7.5 (PBS), fixed for 15 min in absolute ethanol, hydrated in graded alcohol series and finally washed in PBS. They were then flooded for 30 min with a dilution of specific Dugbe virus antiserum, rinsed in PBS and flooded with the conjugate for a further 40 min. After thorough rinse in PBS they were mounted in buffered glycerol. The appropriate controls for uninfected cultures and normal serum were included.

Growth curve. Infected cultures were sequentially harvested and titrated for virus using a micro-culture plaque assay [1]. The distribution of viral inclusion bodies was scored with time.

Results

Cytology. There were no gross cytopathic changes; the infected cell sheet (Fig. 1b) was normal as compared to the uninfected control cultures (Fig. 1a). The only striking feature of the infected cells was the presence of large, paranuclear, basophilic, cytoplasmic inclusion bodies. Most cells contained two of these inclusions, situated on either pole of the nucleus; in a few cells the inclusion surrounded most of the nucleus. In younger cultures (2–3 days), only tiny inclusion bodies were seen, and they grew progressively larger with time.

In a few experiments conducted with LLC-MK2 cells (Rhesus monkey kidney) two further features were observed. In these cultures some occasional cells had a single large nucleus. The other feature was the presence of multinucleated giant cells. These were observed in both uninfected (Fig. 1c) and infected (Fig. 1d) cultures; in the latter they contained huge inclusions situated bipolarly or surrounding the nucleus.

Cytochemical staining. Figs 2a and 2b show the results of methyl-green pyronine staining. The infected cell sheet did not show any gross destruction. The inclusion areas were pyronine positive. Some cells in mitosis also contained inclusion bodies. Feulgen staining failed to show virus-associated features. The features observed by cytochemical staining were confirmed by simultaneous staining of duplicate slides with May–Grünwald-Giemsa.

Immuno-fluorescence. Dugbe virus antigen was confined to the cytoplasm (Fig. 3); the inclusion areas being the most intensively stained. There was also a significant nuclear membrane fluorescence.

Phases of inclusion formation. Cultures were harvested sequentially and stained with May–Grünwald-Giemsa. The various forms of inclusion areas were closely observed, and from the results of several experiments the following picture of inclusion formation emerged. First, there was strong staining of the nuclear membrane; this was followed by paranuclear specks of inclusion areas, these later coalesced to form larger inclusions; or if there were only two bipolar specks of inclusion areas in the first instance, these progressively grew larger. Sometimes in later cultures the inclusion bodies occupied a greater portion of the cytoplasm. The various phases of inclusion formation are diagrammed in Fig. 4.

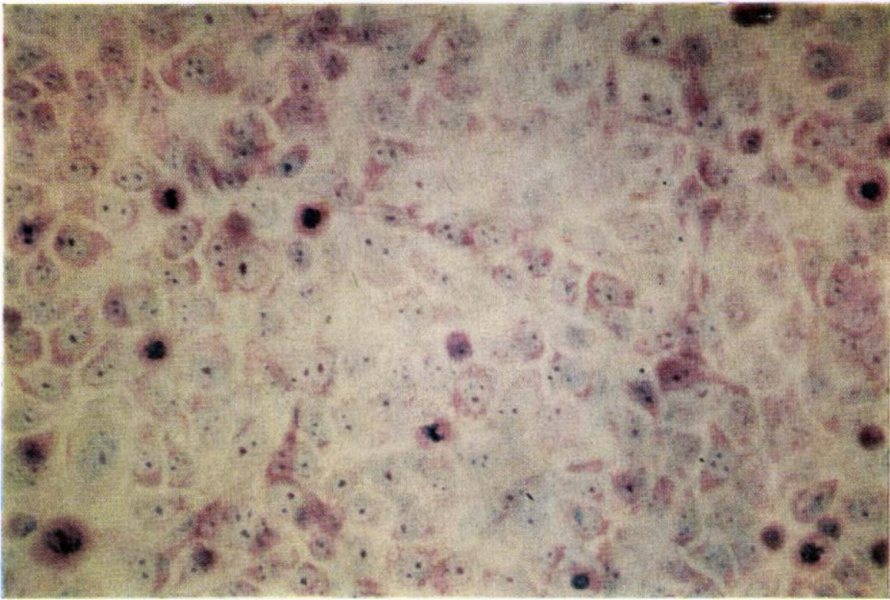


Fig. 2a. Uninfected PS cells (6 days) stained with methyl-green pyronine $\times 400$

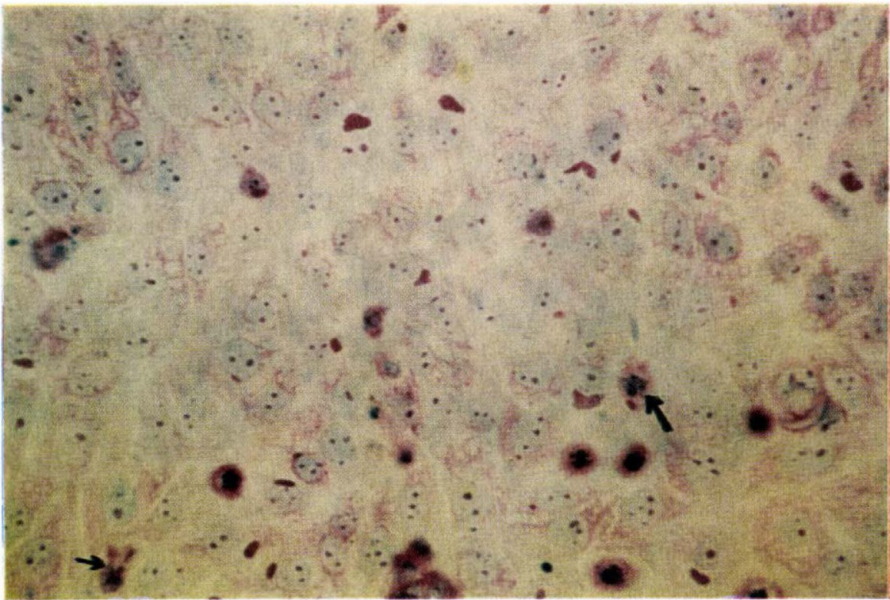


Fig. 2b. PS cells infected with Dugbe virus (6 days) stained with methyl-green pyronine. Note inclusions, cells with inclusions, as well as mitotic figures (arrow). $\times 400$

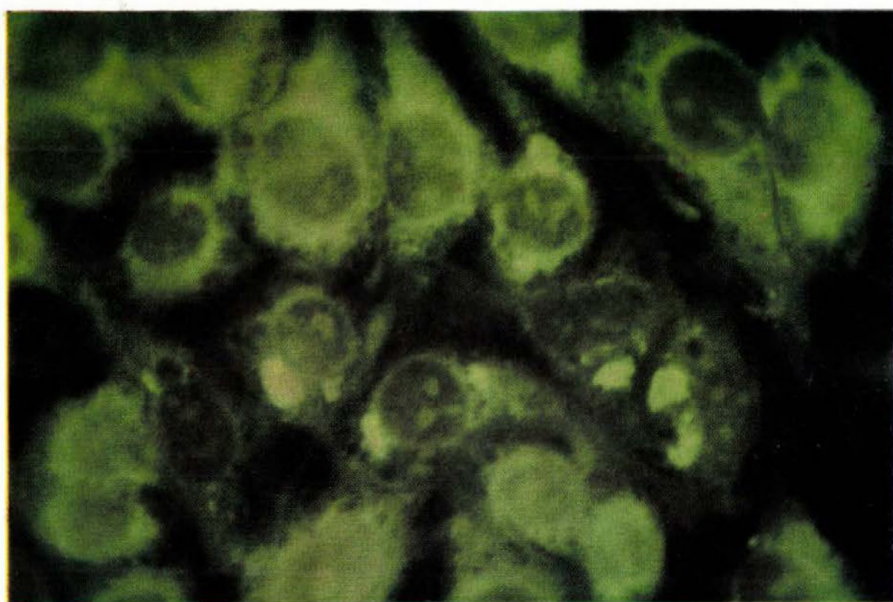


Fig. 3. PS cells infected with Dugbe virus, stained for immunofluorescence. Note exclusive cytoplasmic fluorescence and intense staining of inclusion area. $\times 400$

DUGBE VIRUS - Phases of Inclusion Formation

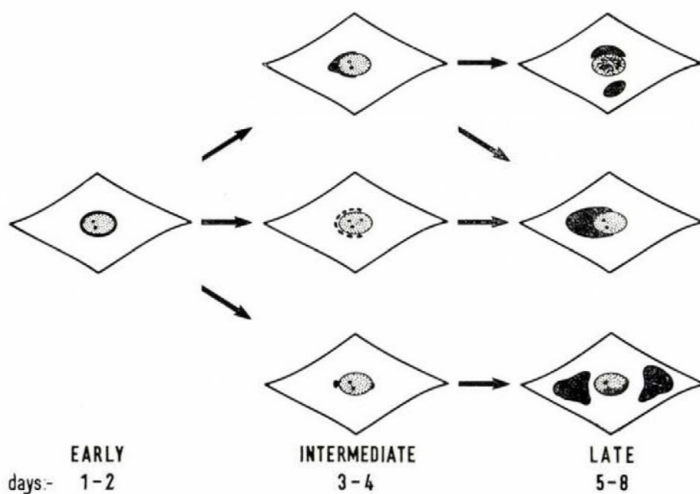


Fig. 4. Diagram of phases of inclusion body formation by Dugbe virus. $\times 400$

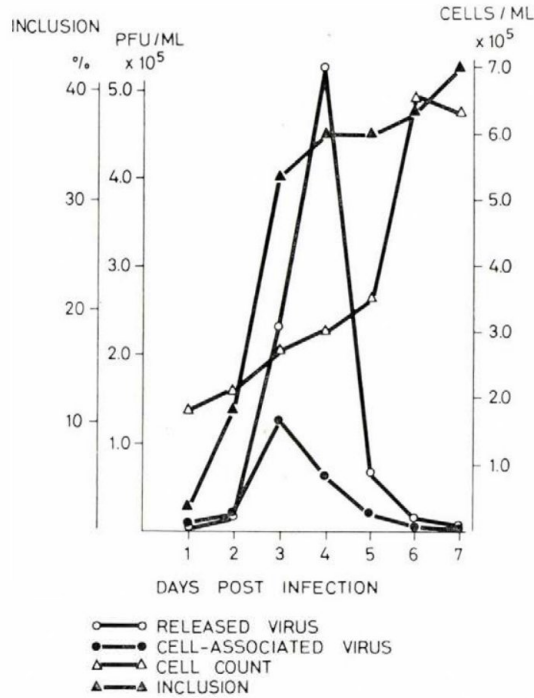


Fig. 5. Growth curve of Dugbe virus in PS cells. $\times 400$

Growth curve. Fig. 5 shows the growth curve of Dugbe virus in PS cells. Four aspects of the growth curve should be stressed. First, most of the virus formed was released from the cells. Second, there was a rapid inactivation of released virus. Third, the infected cells continued to undergo mitotic divisions. Fourth the number of cells with inclusion bodies increased progressively throughout the growth cycle.

Experiments with Dugbe virus strain 1b. Ar. 2484. The results obtained with this strain were identical with those reported above for the prototype strain.

Discussion

The main features of the cytopathology of Dugbe virus in porcine or monkey kidney cells were as follows. The cytopathology was slight and the infected cells seemed to undergo normal mitotic divisions. The formation of intracytoplasmic basophilic inclusion bodies was the most prominent aspect of the cytopathology. It was shown by the immunofluorescent staining that the inclusion areas were depots of Dugbe virus antigen concentration. However, from the growth curve experiments a negative correlation between the titre

of infective virus and the number of cells containing inclusion bodies was demonstrated at the later stages of the growth curve. This would suggest that the inclusion bodies were not a stage in the morphogenesis of the virus. The results of pyronine staining suggested that the inclusion bodies contained ribonucleic acid. Furthermore, it has been concluded from the combined results of the fluorescent antibody staining and the methyl-green pyronine staining that the inclusion bodies are concentrations of viral ribonucleoprotein, possibly noninfective aberrant virus, as suggested by the terminal stages of the growth curve. The inclusion bodies reported here were morphologically similar to those described for influenza B viruses [6], and for mumps virus [7]. In neither case did the inclusion areas contain viral antigen. The latter types of inclusion bodies might therefore be manifestations of non-specific cellular reaction to the infection; indeed, NASIBOV and SMORODINTSEV [7] suggested that such inclusion bodies represented some form of non-specific cellular defence mechanism.

Dugbe virus was said to be remotely related to Ganjam virus from India, by complement-fixation test (CASALS: personal communication). PAUL and DANDAWATE [8] reported that Ganjam virus produced severe cytopathology with polykaryocytosis in Vero (African green monkey kidney) cells. But Dugbe virus differed from Ganjam virus in that it produced slight cytopathology in Vero cells [9] and there was no polykaryocytosis. It should be mentioned that in the present studies a "false" giant cell formation was observed in experiments conducted with the LLC-MK2 (Rhesus monkey kidney) cells (Fig. 1d); but this was not virus specific or virus induced since such cells were also observed in the uninfected controls (Fig. 1c).

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CHROMOSOME ABERRATIONS IN CELLS INFECTED WITH VARIOUS STRAINS OF MEASLES VIRUS

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Summary. The incidence of chromosome fragmentation and/or breakage has been studied in cultured human embryonic diploid cells infected with different wild and vaccinal strains of measles virus. It seemed that for wild strains the mean incidence of fragmentation was two times higher, that of breakage two times lower than for the vaccinal strains.

The ability of both wild and vaccinal strains of measles virus to produce chromosome aberrations has been demonstrated *in vivo* and *in vitro* [1–6]. The occasional ambiguity of the results *in vitro* may be ascribed to the use of various cell systems. It seemed therefore of some interest to compare the incidence of chromosome aberrations in one and the same cell line infected with different strains of measles virus.

Materials and methods

Virus strains. The two lines of strain Leningrad 16 were obtained from batches No. 021–1969 (L16-69) and No. 151–1971 (L16-71) of the Russian type attenuated measles vaccine. Strain Leningrad 4A was kindly supplied by Professor A. A. SMORODINTSEV (Leningrad). Strain E-Bg is a passage line of the Edmonston strain maintained in the National Institute of Public Health (Budapest) and obtained originally from the Department of Virology of the Institute of Hygiene, Belgrade. The Philadelphia (Ph) strain was supplied by the Division of Immunological Producing Control, Medical Research Council Laboratory, London. Strain OKI-II was isolated by us from the leukocytes of a child with acute measles. All the strains listed were adapted to, and maintained on, the permanent monkey kidney cell line PMK III/1 [7].

Human cell line. The human cell line (HEC 684), developed from a 10 weeks embryo obtained at artificial abortion was used for chromosome studies. The line exhibited a normal diploid chromosome complement.

Infection of HEC 684 cells. Cells in the 12th passage were infected, two bottles each with 10^3 to $10^{3.5}$ infectious units per bottle, respectively, of one of the virus strains studied. Colcemid was added after 48 hr incubation. At this time no signs of cell fusion were detectable. The cells were processed for chromosome preparation after incubation with colcemid for one and a half hours.

Chromosome preparations. The chromosomes were prepared by the conventional air drying method.

Results

The chromosome aberrations most frequently found in cells infected with measles virus are breakage and pulverization or fragmentation. Formation of gaps did not seem to be a satisfactorily reliable sign of viral effect [8], therefore this aberration was not registered. Since in the system studied no cell

fusion was detectable at the time of preparation, the possibility of chromosome damage by cell fusion asynchron only with mitosis could be excluded. We therefore prefer to use the term chromosome fragmentation rather than pulverization. The morphology of what we called breakage or fragmentation is shown in Fig. 1 together with some other interesting chromosome aberrations found occasionally in our preparations.

The incidence of breakage or fragmentation in HEC 684 cells infected with different wild and vaccinal strains of measles virus is shown in Table I.

Table I
Incidence of chromosome aberrations in human diploid fibroblasts

No.	Virus strains		No. of mitoses analyzed	Chromosome aberrations, per cent				
	Sign	Type		fragmentation	sum	breakage	sum	total
1	L16-71	vaccinal	149	2.6	12.2	8.0	9.9	22.1
2	L16-69		173	4.6		—		
3	L4-A		217	5.0		1.9		
4	E-Bg		107	8.9		—		
5	Ph	wild	78	11.5	23.6	1.3	4.5	28.1
6	OKI-II		126	3.2		3.2		
7	None		63	—		1.6	1.6	1.6

Although the total incidence of chromosome aberrations was closely similar for the vaccinal strain L16-71 and the wild strain Ph, the former caused mainly breakage while the latter mainly fragmentation. The other two vaccinal strain induced on intermediate incidence of fragmentations but practically no breakage. The wild strain E-Bg caused about a twofold incidence of fragmentation as compared to the vaccinal strains. The wild strain OKI-II appeared to be intermediate insofar it caused fragmentation and breakage with about the same frequency.

Discussion

The results shown in Table I are insufficient for allowing conclusions as to the possible use of the total incidence of chromosome aberrations to distinguish between wild and vaccinal strains grown in one and the same cell type. It seems, however, that the mean incidence of fragmentation is about two times higher and that of the breakage two times lower in the wild than in the vaccinal strains. Thus, wild strains are apparently more liable than the vaccinal one to cause a high incidence the more serious chromosome aberration.

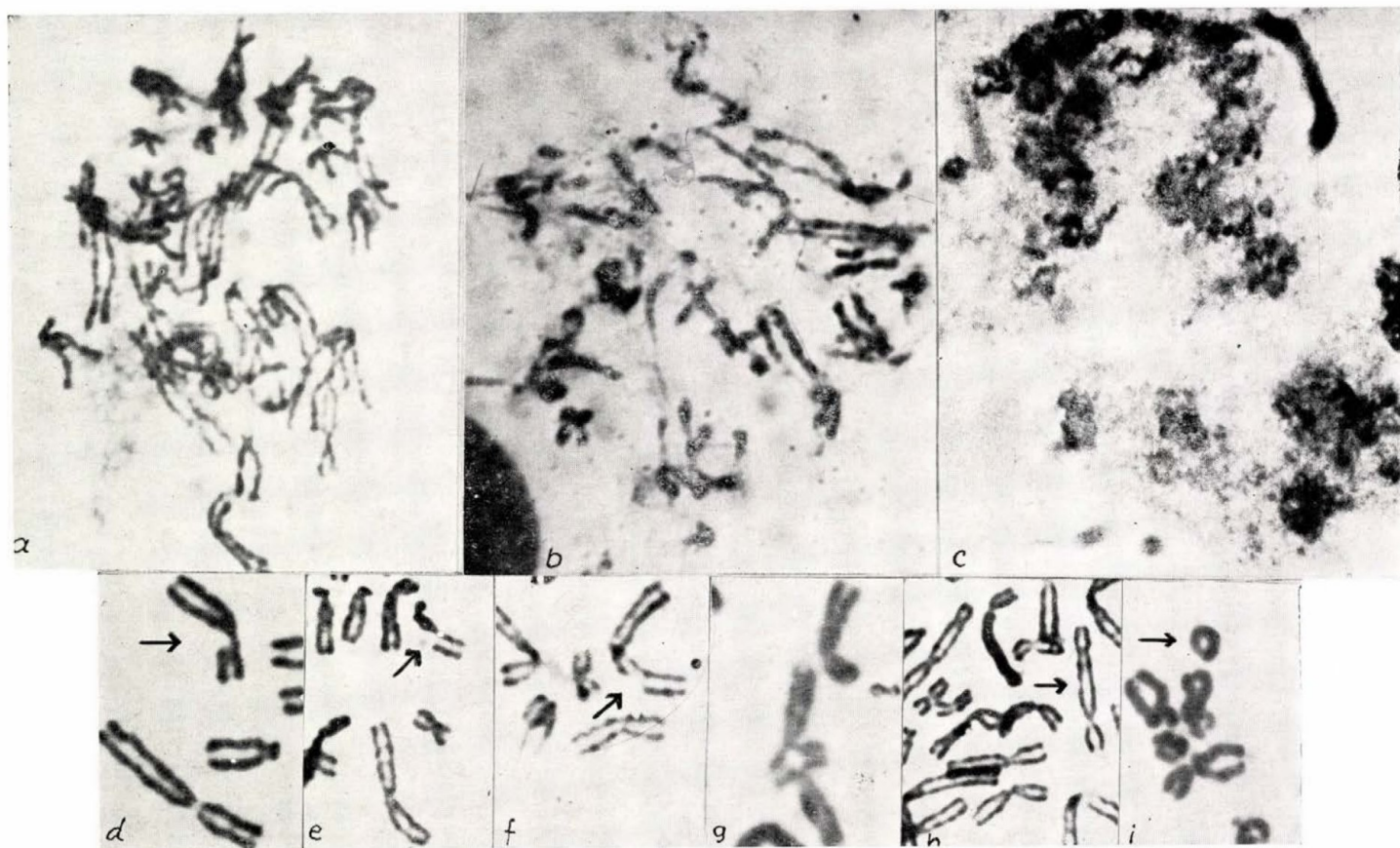


Fig. 1. Types of chromosome aberration found in HEC cells infected with measles virus. a-b-c: different phases of chromosome fragmentation; d-e-f: breakage; g: quadriradial chromosome exchange; h: dicentric chromosome; i: ring chromosome

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DIRECT ISOLATION OF MYCOPLASMAS AND ACHOLEPLASMAS FROM SERA AND KIDNEYS OF CALVES

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Summary. Kidneys and sera of calves from various farms and primary kidney tissue cultures were tested for mycoplasma and acholeplasma contamination. Altogether 24 strains belonging to *Mycoplasma arginini* and *Acholeplasma axanthum* were isolated from tissue cultures, kidneys and sera. The same species were detected from lungs and peribronchial lymph nodes of calves, together with *A. laidlawii*, *A. modicum* and *M. bovirhinis* species. There was a close correlation between the mycoplasma content of tissue cultures, sera and kidneys and the clinical picture observed in the farm, as well as between the quality of tissue cultures and the mycoplasma content of organs, sera and tissue cultures.

Since the first paper [1] reporting contamination of tissue cultures by mycoplasma had been published, it has become evident that a great number of tissue cultures used in biological studies are contaminated with these microorganisms [2, 3]. Established cell lines are much more contaminated than primary tissue cultures [4]. Mycoplasmas can affect the morphology of tissue cultures, producing cytoplasmic granularity, retardation of growth [5], expressed cytopathology, lysis of whole cells [6], transformation of cells [3], margination of chromatin and nuclear segregation and various chromosomal aberrations [5].

The cytopathogenic effect is probably due to the depletion of arginine, or a decrease of glutamine in the medium [7–9], to utilization of nucleic acid precursors and interference with cellular protein and nucleic acid synthesis [10]. As a result, a significant alteration in virus synthesis was observed in mycoplasma-infected cells manifesting itself with an inhibition of replication of viruses such as adenoviruses [11], measles virus [12], Rous sarcoma virus [13] etc. Some authors observed an enhancement of synthesis, for example of respiratory syncytial virus [14], fowl pox virus [15], vesicular stomatitis virus [16].

These facts led to mistakes in virological work [17, 18]. It is therefore important to discover the sources of tissue culture contamination.

Initially it was thought that the sources of contamination were the personnel working with the cell cultures, since the majority of the mycoplasmas isolated were identified as of human origin [2]. However, in recent years mycoplasmas of various animal species have also been isolated [19]. Sera and trypsin

Table I
Isolation of mycoplasmas from calves from farm No. 1

Calf No	Specimens examined	Mycoplasma isolation (strain No.)	Tissue cultures examined	Mycoplasma isolation (strain No.)
1	serum	—	kidney	—
			testicle	—
2	—	—	kidney	—
			testicle	—
	lung	—	kidney	—
3	peribronchial lymph node	—	testicle	—
	kidney	—		
	serum	—		
	lung	—	kidney	—
4	peribronchial lymph node	—	testicle	—
	kidney	—		
	lung	—	kidney	227
5	peribronchial lymph node	233	testicle	—
6	lung	—	kidney	—
	kidney	—	testicle	—
	serum	—		
7	—	—	kidney	—
			testicle	—
	lung	—	kidney	—
8	peribronchial lymph node	282	testicle	—
	kidney	—		
	lung	—	kidney	—
9	peribronchial lymph node	309	testicle	—
	kidney	310		

cal symptoms of respiratory disease. The calves examined were clinically healthy but some of them had a small area of catarrhal pneumonia.

From 8 animals from farm No. 3, 1 lung, 1 peribronchial lymph node, 8 kidneys, 6 sera, 6 kidney tissue cultures and 1 testicle tissue culture were tested. No mycoplasma was detected in the specimens. It was possible to establish primary cell cultures from the kidneys. All animals in farm No. 3 were healthy.

There was a correlation between isolation of mycoplasmas from serum and kidney and the quality of the primary tissue cultures (Table III). If mycoplasmas were present in the kidney and serum, mycoplasmas were present in the tissue cultures, too. In this case, the quality of the cell culture was not satisfactory.

Table II
Isolation of mycoplasmas from calves from farm No. 2

Calf No.	Specimens examined	Mycoplasma isolation (strain No.)	Tissue cultures examined	Mycoplasma isolation (strain No.)
10	lung	—	kidney	350
	peribronchial lymph node	—	testicle	356
	kidney	—		
	serum	351		
11	lung	—	—	
	kidney	—		
	serum	371		
	lung	380	—	
12	peribronchial lymph node	—		
	kidney	—		
	lung	—	—	
13	peribronchial lymph node	391a		
		391b		
	kidney	381		
	lung	—	kidney	408
14	peribronchial lymph node	—		
	serum	415		
	lung	407a	—	
15		407b		
	serum	413		
	lung	—	—	
16	peribronchial lymph node	—		
	kidney	478		
17	kidney	—	kidney	487
17	serum	—	testicle	495
18	kidney	+ (not identified)	kidney	+ (not identified)
	serum	+ (not identified)	testicle	+ (not identified)
19	kidney	499	kidney	501
	serum	500		

Table III
Mycoplasma isolation and cytopathic effect in primary tissue culture

Mycoplasma isolation from serum and kidney	Cytopathic effect in primary tissue cultures	
	positive	negative
Serum and/or kidney positive	7	1
Serum and kidney negative	1	11

Serological examination of strains isolated from animals of farm No. 1 revealed 4 strains belonging to *A. axanthum* and 1 strain to *A. laidlawii*. *A. axanthum* was present in tissue cultures but this species occurred in the peribronchial lymph node together with *A. laidlawii*. Among the strains cultivated from calves of farm No. 2, two strains were identified as *A. modicum*, two strains as *M. bovirhinis*, 15 as *M. arginini* (Table IV). In this group of

strains from tissue cultures, sera and kidneys *M. arginini* was only detected. Other species were cultivated from the lungs and peribronchial lymph nodes, which also contained *M. arginini*.

Table IV
Serological examination of the isolated strains by growth inhibition test
Size of inhibition zones in mm

Strain No.	Antisera					
	<i>A. laidlawii</i> A (PG8)	<i>A. granularum</i> (Friend)	<i>A. modicum</i> (Squire PG49)	<i>A. axanthum</i> (ATCC 25176)	<i>A. bovirhinis</i> (PG43)	<i>M. arginini</i> (G230)
227	—	—	—	3.0	—	—
233	—	—	—	2.5	—	—
282	3.0(part)	—	—	—	—	—
309	—	—	—	2.7	—	—
310	—	—	—	2.8	—	—
350	—	—	—	—	—	3.0
351	—	—	—	—	—	2.8
356	—	—	—	—	—	2.6
371	—	—	—	—	—	3.0
380	—	—	—	—	1.5	—
381	—	—	—	—	—	3.2
391a	—	—	—	—	—	3.5
391b	—	—	2.0	—	—	—
407a	—	—	2.0	—	—	—
407b	—	—	—	—	—	3.0
408	—	—	—	—	—	3.8
413	—	—	—	—	—	3.1
415	—	—	—	—	—	2.8
478	—	—	—	—	2.8	—
487	—	—	—	—	—	3.1
495	—	—	—	—	—	3.0
499	—	—	—	—	—	3.2
500	—	—	—	—	—	3.5
501	—	—	—	—	—	3.5

Discussion

The present results demonstrated a significant correlation between the ability to establish primary tissue cultures from kidney and the presence of mycoplasma in the tissue cultures, as well as in the serum and kidneys used for preparation of tissue cultures. In the presence of mycoplasma, the quality

of tissue cultures was not satisfactory. Divergences in the experiments might be explained by the concentration of mycoplasmas having been too low for destroying the cells (calf No. 12) or for isolation from serum and kidneys (calf No. 17) at the time of examination.

Identification of the isolated strains revealed that all were belonging to bovine species [22]. *M. arginini* and *A. axanthum* were isolated from kidneys, sera and tissue cultures. These species occurred in the lung and peribronchial lymph nodes together with *M. bovirhinis*, *A. modicum* and *A. laidlawii* species. The results showed some relationship between the epizootological picture in the farms and the mycoplasma species detected in sera and kidneys. Farm No. 3 was free from respiratory disease, and no mycoplasma and acholeplasma were present in the sera and kidneys. In farm No. 1, slight symptoms of respiratory disease were observed. *A. axanthum* occurred in kidney and tissue culture, and also in the respiratory organs. The most severe respiratory disease was noticed in farm No. 2. Mostly *M. arginini* occurred in the kidneys, sera and in the respiratory organs, too. During the disease, mycoplasmas may have reached the blood and several organs including the kidneys. Mycoplasmas could be isolated from sera and kidneys of animals slaughtered at this period of time.

Our data agree with those of BARILE and KERN [20] concerning the presence of *M. arginini* in sera used for tissue cultures. According to our results, organs used for primary tissue culture preparation may also be contaminated with *A. axanthum*. This species was first isolated from tissue cultures [24]. The present data have confirmed the report of AL-AUBAIDI and FABRICANT [25], concerning the occurrence of this species (serogroup K) in bovine sources.

On the basis of our observations it may be assumed that mycoplasma contamination of tissue cultures is connected with the mycoplasma content of sera and organs used for preparation. Thus for this purpose sera and organs should be collected from healthy animals free from respiratory disease, especially from mycoplasmas and acholeplasmas pathogenic for animals. Sera obtained from animals of various farms should be collected and prepared separately. It is also advisable to check each batch of serum and tissue culture for mycoplasma and acholeplasma contamination.

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ELECTRON MICROSCOPY OF PHAGES LIBERATED BY MEGACIN A PRODUCING LYSOGENIC BACILLUS MEGATERIUM STRAINS

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Summary. Mitomycin C was added at fairly high concentration (5–10 $\mu\text{g/ml}$) to exponentially growing cultures of selected strains of *Bacillus megaterium*. Lysis of the bacteria followed, associated by liberation of phage and megacin A production. In contrast, a low concentration (0.5 $\mu\text{g/ml}$) of mitomycin induced only megacin A production. Electron microscopic examination of the lysates induced by 5–10 $\mu\text{g/ml}$ of mitomycin in 19 strains of *B. megaterium* showed them all to contain phages; most of the strains proved polylysogenic. Their lysates contained distinct complete phages of different structures and dimensions. A few strains released defective phage particles. The significance of the electron microscopic findings is discussed in relation to megacinogeny.

Several papers have reported on an antibacterial substance designated megacin A, produced by *Bacillus megaterium* [1]. Megacins can be classified into at least three different groups [2]. Megacin A formed by 2–3% of *B. megaterium* strains isolated from soil samples exhibits a normal growth curve when cultivated in an adequate complete nutritional medium. No megacin A formation could be detected in the cultures unless the bacteria had been exposed to an agent disturbing DNA synthesis. On light ultraviolet irradiation of the growing cells, or addition of certain chemicals such as mitomycin C or nitro-soguanidine to their cultures, a marked lysis ensues, accompanied by megacin A production [3]. Megacinogeny is reminiscent of lysogeny, except that there is no infective phage, but an antibacterial substance is released during lysis of the cells. The resemblance of the two phenomena suggested that a prophage might be involved in both cases. Although it has been claimed that the two phenomena are related genetically [1, 4], no direct evidence could be obtained which supported this concept. On the other hand OZAKI *et al.* [5] succeeded in proving the enzymic nature of megacin A; the bacteriocin of strain 216 was shown to be identical with phospholipase A. Unfortunately, other strains produce too little megacin A for allowing its identification. There is, however, no doubt that different strains produce the same megacin A [1]. A turning point has recently been reached when a *Bacillus cereus* strain was found to be capable of producing a bacteriocin similar to megacin A; chemical evidence indicated that this bacteriocin is identical with phospholipase A [6]. This particular strain of *B. cereus*, designated W, is lysogenic for a highly specific phage which attacks all strains of *B. anthracis* [7]. Furthermore, another

prophage was also detected in strain W, controlling the liberation of a distinct phage, named *wx*. This particular phage did not propagate when tested on a great number of strains belonging to different species of the genus *Bacillus*. The only host which appeared sensitive to phage *wx* was a "cured" derivative, obtained from strain W of *B. cereus*, and not producing megacin A, i.e. phospholipase A [8]. Infection of these "cured" bacteria by phage *wx* converted the cells into phospholipase A producers. This was direct evidence of megacin A production being due to a certain prophage [9].

In the present study our aim was to examine lysates derived from megacinogenic strains of *B. megaterium* after induction, for the presence of either complete or defective phage particles.

Materials and methods

Bacterial strains. Out of a total of 18 megacin A producing strain, 10 were isolated from soil samples up to 1954 [10], and 8 further strains have recently been screened from about 150 isolates of *B. megaterium*. They were identified on the basis of the criteria established by SMITH et al. [11]. Strain No. 337 had been received from the collection of Dr. DEN DOOREN DE JONG [12]. Without induction this strain produced a "killing principle" [13], which was identified as a protein and designated as megacin Cx [14]. Strain MUT-C was a phage-resistant one, although highly sensitive to megacin A.

Medium OX. Trypton (Oxoid), 10 g; yeast extract (Oxoid), 2.5 g; K_2HPO_4 , 1 g; NaCl, 5 g; distilled water, 1000 ml; pH 7.5. Solid medium was prepared by adding 1.5% agar to OX broth.

Induction of megacin A. A liquid culture was aerated by shaking in a water bath at 37°C, and its growth was monitored by a Bausch and Lomb Spectronic 20 optical densitometer. When bacteria were induced to megacin A production, 0.5 µg/ml of mitomycin C (MC) was added to the culture. Further technical details of the induction and of the titration of megacin have been described previously [15].

Titration of megacin A. Preparation of the indicator plate has been described previously [15]. Several twofold dilutions of the lysate were prepared in saline solution containing 20% OX broth. By means of a loop, 0.02 ml amounts of the serial dilutions were placed onto the surface of the indicator plates. One unit of megacin A/ml was taken as the reciprocal of the highest dilution which gave either a clear zone or lysed at least half of the bacteria.

Induction of the prophage. One volume of a 18 hr broth culture of the strain under investigation was added to ten volumes of freshly prepared OX medium and incubated on a shaker to a titre of 5×10^6 cells per ml. Then mitomycin C was added to a concentration of 5–10 µg per ml and incubation was continued. After 3–4 hr the suspension clarified somewhat and was centrifuged first at 6000 g and then at 100 000 g in a Hans Janetzky VAC-60 apparatus. The pellet was resuspended in as little saline as possible and centrifuged again at 6000 g.

Electron microscopy. The purified phage preparation was dialyzed against distilled water and placed on carbon-impregnated Formvar-film stained with 1% uranyl acetate and examined in a JEM-7 electron microscope at 80 kV.

Results

Liberation of phage particles by megacinogenic strains of *B. megaterium*. Although maximum production of all megacinogenic A strains could be induced by the addition of 0.5–1 µg/ml of MC, phage liberation could be induced only by MC at a concentration of 5–10 µg/ml. Altogether 4 apparently morphologically distinct, and 2 defective phages were detected in the lysates of 18

individual strains of megacin A producing *B. megaterium*. Table I shows the distribution of the different phages produced by the individual strains. Electron microphotographs of the preparations made of concentrated lysates of a number of *B. megaterium* strains revealed that all of them contained either complete bacteriophages of different structure and dimension, or defective phage particles (Table I, Figs 1-5). Different strains of *B. megaterium* could release from one to five kinds of phage. Strain 704 produced five different bacteriophages. This was apparently a polylysogenic culture carrying several genomes of morphologically complete as well as defective phages. This was consistent with the date of HENNEBERRY and CARLTON [16]. They isolated several circular DNA molecules from the bacterial genome of the *B. megaterium* strain 216 which they believed to be the genomes of some temperate phages. It should be noted that polylysogeny is frequently found among different strains and species of microorganisms (RAUTENSTEIN [17]).

Ultrastructure of the phages produced by B. megaterium. Bacteriophages released by different strains of *B. megaterium* after induction are morphologic-

Table I
B. megaterium phages obtained by mitomycin C induction

Strains	Designation of phage					Total	Megacin A, unit/ml
	1	2	3	4	5		
216		+	+	+		3	40 000
508			+			1	100
575			+			1	200
588		+	+	+		3	400
594				+		1	100
604		+	+			2	100
637		+				1	200
638		+		+		2	300
659		+				1	100
689	+	+	+			3	200
701	+	+		+	+	4	200
704	+	+	+	+	+	5	400
717		+	+	+	+	4	100
743				+	+	2	400
791	+	+				2	400
807 ^a	+	+	+			3	100
807		+	+			2	400
842				+		1	400
337		+		+		2	0

ally different. *B. megaterium* produced phages belonging to practically every morphological group classified by BRADLEY [18] and TIKHONENKO [19]; they were designated as phages 1, 2, 3, 4, 5. Phages 1–4 were complete phages particles, while phage 5 appeared to be a defective one having a tail without a head.

Phage 1 has a head measuring 120 nm in diameter and a tail 270 nm in length, terminating in an easily discernible base plate. The phage has a contractile tail sheath. The contracted sheath is shortened by about half its length and is approximately 140 nm. The base plate of phage 1 has a complex structure and is much wider than the tail itself (Fig. 1).

Phage 2 belongs to a group of phages with contractile sheath. It is however, much smaller than phage 1. The head of the phage is only 60 nm in diameter and the tail is 110 nm long. The tail ends in a small base plate (Fig. 2).

Phage 3 belongs to the group with non-contracting tail. Its head is a hexagon of 55 nm in diameter and its non-contracting tail shows a well defined striation. The base plate is rather small (Fig. 3).

Phage 4 particles have a head 65 nm in diameter and a very short tail. The tail actually consists of a base plate connected by a very short neck with the head (Fig. 4).

Phage 5 resembles the extended tail of a T-even bacteriophage. Its particles are actually headless tails ending in a small base plate with thin fibres (Fig. 5). Thus, the particles of this phage have all the structural elements of the normal phage tail. Structures of this kind are well-known to be produced by ISHII [20] and pyocin isolated by TAUBENECK [21] and extensively studied by TIKHONENKO *et al.* [22] and by POPOVA *et al.* [23]. Thus, all strains of *B. megaterium* release phage particles as shown by electron microscopy. It should be added that the presence of biologically inactive defective particles can be detected only by electron microscopy.

Of all the described phages the most frequent was phage 2 which was produced by 14 strains out of 19, and particles bearing a morphological likeness to phage 5 were released by 10 out of 19 strains (Table II).

All strains except strain 337 produced megacin A. It is not known whether there is a connection between megacin A and the defective phages released by *B. megaterium* after induction.

Fig. 1. A particle of phage 1 with extended tail sheath; bacterial flagella indicated by arrow.

Uranyl acetate ($\times 250\,000$)

Fig. 2. Particles of phage 2 with extended and contracted tail sheaths. Uranyl acetate ($\times 250\,000$)

Fig. 3. A particle of phage 3 with non-contracting tail. Uranyl acetate ($\times 250\,000$)

Fig. 4. Particles of phage 4, with short tail indicated by arrow. ($\times 250\,000$)

Fig. 5. Particles of defective phage 5 structurally resembling the tail of T-even phages.

Uranyl acetate ($\times 250\,000$)

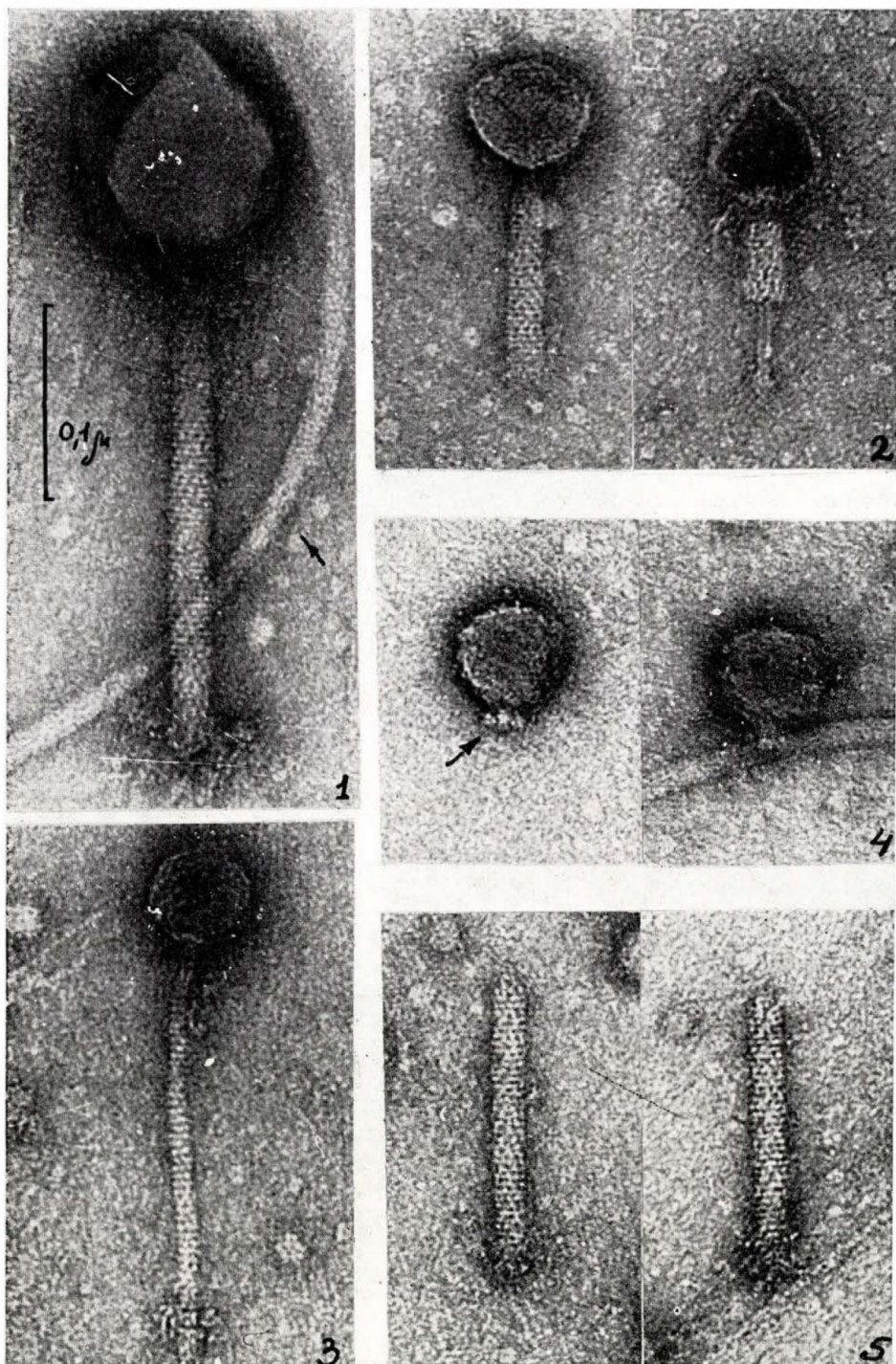


Table II
*Dimensions of B. megaterium phages obtained by
mytomycin C induction*

Phage No.	Dimension of head, nm	Dimension of tail, nm
1	120	270
2	60	110
3	55	210
4	65	—
5	—	160

Discussion

The presence of phages has been revealed by electron microscopy in the lysates of all the strains investigated. They, except No. 337, produced megacin A and the addition of mitomycin C induced a marked lysis accompanied by an accumulation of megacin A in the culture. Strain No. 337 was not lysed significantly, but the culture ceased growing at the middle of the exponential phase, and the arrest of growth was probably due to the presence of MC at high concentration (5 $\mu\text{g/ml}$). No traces of megacin A were found in the supernatant of the culture of No. 337; this strain is known to produce megacin Cx [14] instead of megacin A. Nevertheless, electron microscopy revealed two phages (PH 2 and PH 5) in its supernatant.

It is difficult to conclude to any correlation between megacin A production and the phages liberated by induction. The morphological features of phages are not related to any biological characteristics. The present results indicate that all the investigated *B. megaterium* strains harbour a few prophages. They have been detected by electron microscopy, while earlier attempts to reveal any phage released by strain 216 had failed [24]. Thus, when the bacteria were exposed to light ultraviolet irradiation and a preparation was made with Kellenberger's technique for electron microscopy, although the gradual lysis of the bacteria could be followed, neither mature phage particles nor structures reminiscent of some incomplete form of phage were found around the cells during different stages of the lysis. Investigations are in progress to isolate and propagate individual phages liberated by megacinogenic strains of *B. megaterium* in order to study whether any of these phages is capable of converting a negative strain to a megacinogenic one.

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СЕРОЛОГИЧЕСКОЕ ОБСЛЕДОВАНИЕ ЖИВОТНЫХ В ВЕНГРИИ НА АНТИТЕЛА К ВИРУСУ КРЫМСКОЙ ГЕМОРРАГИЧЕСКОЙ ЛИХОРАДКИ

Л. Б. ХОРВАТ

Государственный Институт Здравоохранения, Будапешт

(Поступила 30 мая 1974 г.)

L. B. HORVÁTH (*National Institute of Public Health, Budapest*): **Incidence of antibodies to Crimean haemorrhagic fever in animals.** Sera from 687 cattle and 48 sheep were collected in eight different counties and examined with agar gel diffusion technique for the presence of Crimean haemorrhagic fever antibodies. Antibodies were detected in 21 animals (6 cattle and 15 sheep). It has been concluded that the possibility of occurrence of Crimean haemorrhagic fever in Hungary should be considered.

Резюме

С целью обнаружения антител против крымской геморрагической лихорадки исследовано 687 коровьих и 48 бараньих сывороток, собранных из 8 областей Венгрии, в реакции диффузионной преципитации в агаре. Антитела обнаружены в 6 случаях у крупного рогатого скота и в 15 случаях исследования сывороток мелкого рогатого скота. На основе этих результатов предполагается присутствие крымской геморрагической лихорадки в Венгрии.

До сих пор мы не имели сведений о наличии крымской геморрагической лихорадки (КГЛ) в Венгрии [1–3]. Однако, на территории Венгрии имеются места с природными условиями, благоприятными для размножения клещей. После приобретения соответствующего антигена мы провели исследования с целью получения ответа на вопрос: имеется ли КГЛ в Венгрии?

Материал и методы

С августа 1972 года по июнь 1973 года из 8 областей страны были собраны сыворотки от домашних животных: крупного (крс) и мелкого (мрс) рогатого скота. Сыворотки, поступившие в лабораторию, были инактивированы при +56°C и содержали 4% борной кислоты.

В качестве антигена применяли сахарозо-ацетоновый антиген, приготовленный из мозга новорожденных белых мышей, зараженных штаммом «Пятн.» вируса КГЛ.

Сыворотки исследовали в реакции диффузионной преципитации в агаре (РДПА) по методу Ouchterlony [4]. Применяли 4 единицы упомянутого антигена.

Антиген и контрольную положительную сыворотку получили из Института полиомиелита и вирусных энцефалитов АМН СССР (Москва).

Результаты

Исследовано 687 сывороток крс (коровьих) и 48 сывороток мрс (бараньих). Место, время сбора и результат исследования показаны в таблице № 1.

Среди 687 исследованных сывороток крс 6 оказались положительными в неразведенном виде. Среди 48 сывороток мрс в 15 случаях имелись специфич-

Таблица № I

Исследование сывороток домашних животных в РДПА с КГЛ антигеном

Собранные сыворотки		Результаты	
Место (область)	Время (месяц)	крс	мрс
Боршод-Абауй-Земплен	VIII, III	0/76 ¹	н. и. ²
Чонград	VIII	0/2	н. и.
Саболч-Сатмар	VIII	0/20	н. и.
Зала	VIII	0/4	н. и.
Хайду-Бихар	IX, X, II, VI	1/170	15/38
Бараня	II, V	0/100	н. и.
Солнок	II	0/10	н. и.
Ваш	V	5/305	0/10
Всего		6/687	15/48

Замечание: 1 — числитель: число положительных сывороток, знаменатель: число исследованных сывороток, 2 — н. и.: не исследованы

Таблица № II

Титры преципитирующих антител в сыворотках домашних животных

Собранные положительные сыворотки		крс			мрс		
		титры сывороток: 1:					
Место (область)	Время (месяц)	1	2	4	1	2	4
Хайду- Бихар	II, VI V	1	—	—	10	2	3
Ваш		5	—	—	—	—	—
Всего		6	—	—	10	2	3

ческие преципитирующие антитела к антигену КГЛ. В 2 случаях титр антител был 1 : 2, в 3 случаях — 1 : 4. Место, время сбора и титры положительных сывороток указаны в таблице № 2.

Обсуждение

Инфекционный процесс у крупного и мелкого рогатого скота протекает латентно и завершается образованием специфических антител [5]. Это позволяет использовать животных в качестве индикатора для определения циркуляции вируса КГЛ в природе.

Хотя для разведения баранов импортируют разные виды из Советского Союза, положительная корова из области Хайду-Бихар местного происхождения. Последний факт указывает на возможность встречаемости КГЛ в Венгрии. Но её значение, особенно в вопросе заражения людей, требует дальнейшего исследования.

Благодарность. Выражаю благодарность проф. М. П. ЧУМАКОВУ за ценные научные советы, Т. И. ЗАВОВОЙ за предоставление в наше распоряжение КГЛ антигена и положительной иммунной сыворотки (Институт полиомиелита и вирусных энцефалитов АМН СССР). Выражаю благодарность за сбор исследуемых сывороток БЕЛА НАДЬ (директору ветеринарного института г. Сомбатхей), ЯНОШУ ТАНИ (зав. отдела ветеринарного института г. Дебрецен), ЭРЖЕБЕТ БАНФАЛВИ (замзав. отдела ветеринарного института г. Мишкольца), Ш. Гёне (спец. ветеринарному врачу ветеринарного института г. Сомбатхей), ИМРЕ ТАКАЧУ (спец. ветеринарному врачу скотобойни г. Будапешт), АНДРАШУ НАДОРУ (лаб. спец. ветеринарному врачу ветеринарной станции г. Печ).

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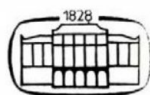
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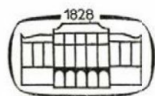
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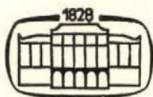
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NATURAL ANTIBODIES IN HEALTHY ADULTS*

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(Received January 22, 1974)

Summary. The serum of 100 adults living in Budapest was examined for isohaemagglutinin titre with haemagglutination, for staphylococcal-antitoxin titre with haemolysis inhibition and for bacterial antibody titre against 17 different groups of bacteria with passive haemagglutination. Antibody levels in males, except for certain bacterial antibodies, were somewhat lower than in females. The antibody titres, especially in men, decreased gradually from 20 to 50 years of age and were usually lower in Rh negative than in Rh positive persons.

Natural antibodies against different bacteria, bacterial toxins, viruses [1-4] and heteroagglutinins acting on sheep erythrocytes [5] have widely been studied. The immunogram developed by BACKHAUSZ *et al.* [6] proved the best for characterizing the immunological status of the person examined. This method, based on the level of certain antibodies present in the majority of the healthy population, has successfully been employed for examining the immunological status of patients with paraproteinaemia, malignant hyperthyroidism [6], agammaglobulinaemia [7], in disturbances following a blood exchange [8], for revealing antibodies against enteric bacterial antigens [9, 10], and in patients with ophthalmic diseases [11, 12].

In the present study, persons living in Budapest were examined with special regard to the association of antibody levels with age, sex and Rh group.

Materials and methods

Serum was taken from 100 healthy blood donors under regular medical control.

Bacterial antigens were prepared in the Institute for Serobacteriological Production and Research Human, Budapest, from the following organism: *Salmonella typhi* O strain 901, *S. typhi* Ty2, *S. typhi* Vi, *S. paratyphi-A* HA 6, *Shigella flexneri* 1b, *S. flexneri* 2a, *S. flexneri* 3, *S. flexneri* 6, *S. sonnei*, *Escherichia coli* O26, *E. coli* O55, *E. coli* O86, *E. coli* O111, *Pseudomonas aeruginosa* O1, O3a, 3d, 3e, O4a, 4b, O5a, 5b, 5d, O7a, 7b, *Vibrio cholerae* serogroups Ogawa and Inaba.

The antigens were prepared by Boivin's trichloroacetic acid method. One ml antigen represented 10^{10} bacterial cells as determined by comparison to the international opacity standard. Purified protein derivative (PPD) was used as *Mycobacterium tuberculosis* antigen. Staphylococcal α -toxin and α -antitoxin (ASTA) were prepared by Human (Budapest).

* Presented in part at the Meeting of the Hungarian Association of Immunology, May 10-12, 1973, Balatonfüred, Hungary

Determination of isohaemagglutinins. The procedure of BACKHAUSZ *et al.* [6] based on TAKÁTSY's micromethod [14] was used. After determining the blood group of the examined person, to a two-fold dilution series of the serum equal amounts (0.025 ml) of a 2% erythrocyte (A or B group) suspension were pipetted. After incubation at room temperature for 1 hr the haemagglutination titre was read in the usual manner.

Determination of bacterial antibodies with passive haemagglutination. The method of BACKHAUSZ *et al.* [6] was employed using TAKÁTSY's plates [13]. Human 0 group erythrocytes were washed twice, resuspended to give a 10% suspension and mixed with equal volumes of bacterial antigens. After incubation at 37°C for 1 hr the erythrocytes were washed twice and resuspended in saline to 2%. To twofold serial dilutions of the sera equal amounts (0.025 ml) of sensitized erythrocytes were given. After incubation at 37°C for 1 hr the results were read.

Determination of staphylococcal α -antitoxin (ASTA) with haemolysis inhibition. The method of SZATMÁRY [14] as modified for microtechnique by BACKHAUSZ *et al.* [6] was used. A standard antitoxin of known titre and the serum specimens examined were diluted serially and after the addition of titrated amounts of staphylococcal α -toxin, the plates were incubated at 37°C for 1 hr, then rabbit erythrocytes washed twice were added. The titre of haemolysis inhibition by the serum specimen was compared to the haemolysis inhibition titre of the standard antitoxin. Antibody content per ml was expressed in international units.

Statistical evaluation. Isohaemagglutinin and bacterial antibody titres were expressed as reciprocals of twofold serum dilutions [6]. Normal levels of antibodies were given as average values ($\bar{x}$) with the standard deviation (SD) [15].

Results

Age, sex and blood groups of the examined persons. Age and sex distribution of the 100 blood donors is presented in Table I. Among them, 55 were Rh negative and 45 were Rh positive.

Table I
Age and sex distribution

Age	Females	Males
20—30	4	22
31—40	8	24
41—50	8	24
51—60	7	3
Total	27	73

AB0 blood group distribution of the persons examined in this study has been compared with the data of BACKHAUSZ *et al.* [15] for healthy adults in Budapest (Table II). It is seen that the persons selected did not reflect the blood group distribution common in Budapest.

Natural antibody levels. Average isohaemagglutinin titres and the standard deviation according to sexes are shown in Table III. The average antibacterial titres against 17 antigens is presented in Table IV. The distribution of bacterial

Table II

*Distribution of AB0 blood groups in adults living
in Budapest according to earlier and
the present data*

Blood groups	Percentage	
	earlier studies*	present studies
0	33.2	36
A	40.9	24
B	18.5	17
AB	7.3	23
Total no. of persons	1168	100

* BACKHAUSZ [16]

antibodies according to sexes is analyzed in Table V. Table VI shows the staphylococcal α -antitoxin titres according to sexes.

The results indicated that antibody levels in men, except for *S. typhi* O, *S. typhi* Vi, *S. typhi* 2, *S. sonnei* and *E. coli* O55 were somewhat lower than in women. *V. cholerae* and PPD antibodies were considerably lower in titre than other bacterial antibodies.

Differences from the average titres for isohaemagglutinins and bacterial antibodies are shown in Fig. 1. The zero line represents the average value for

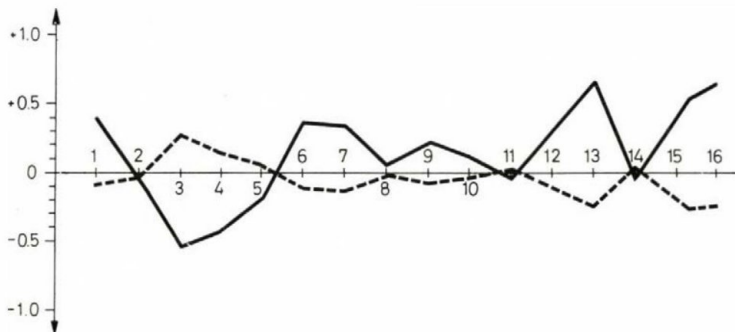


Fig. 1. Difference in average titre according to sex (— 27 women, - - - - - 73 men) from the average titres for 100 persons. 1 = anti-B, 2 = anti-A, 3 = *S. typhi* O, 4 = *S. typhi* Vi, 5 = *S. typhi* 2, 6 = *S. paratyphi*-A, 7 = *S. flexneri* 1, 8 = *S. flexneri* 2, 9 = *S. flexneri* 3, 10 = *S. flexneri* 6, 11 = *S. sonnei*, 12 = *P. aeruginosa*, 13 = *E. coli* O26, 14 = *E. coli* O55, 15 = *E. coli* O86, 16 = *E. coli* O111

Table III*Distribution of isohaemagglutinin titres according to sex*

	Anti-A			Anti-B		
	No.	$\bar{x}$	SD	No.	$\bar{x}$	SD
73 men	39	5.92	1.06	49	5.85	1.57
27 women	14	5.93	0.78	11	6.32	1.75
Total	53	5.92	1.06	60	5.93	1.59

Table IV*Average titre for bacterial antibodies*

Antibody for	No. of persons	$\bar{x}$	SD
<i>S. typhi</i> O901	100	4.81	1.69
<i>S. typhi</i> Vi	100	3.87	1.95
<i>S. typhi</i> 2	100	5.06	1.62
<i>S. paratyphi-A</i>	100	4.03	1.39
<i>S. flexneri</i> 1b	100	5.34	1.40
<i>S. flexneri</i> 2	100	4.84	1.39
<i>S. flexneri</i> 3	100	4.94	1.42
<i>S. flexneri</i> 6	100	6.19	1.47
<i>S. sonnei</i>	100	5.45	1.58
<i>E. coli</i> O26	100	4.49	1.75
<i>E. coli</i> O55	100	3.37	1.63
<i>E. coli</i> O86	100	5.78	1.82
<i>E. coli</i> O111	100	3.74	1.36
<i>P. aeruginosa</i>	100	4.99	1.71
<i>V. cholerae</i> Ogawa	100	0.07	
<i>V. cholerae</i> Inaba	100	0.15	
PPD	100	0.34	

the 100 examined persons. Differences from the average are shown on the abscissae.

Figs 2 and 3 show in a similar manner the differences from the average titres according to the age of female (Fig. 2) and male (Fig. 3) persons.

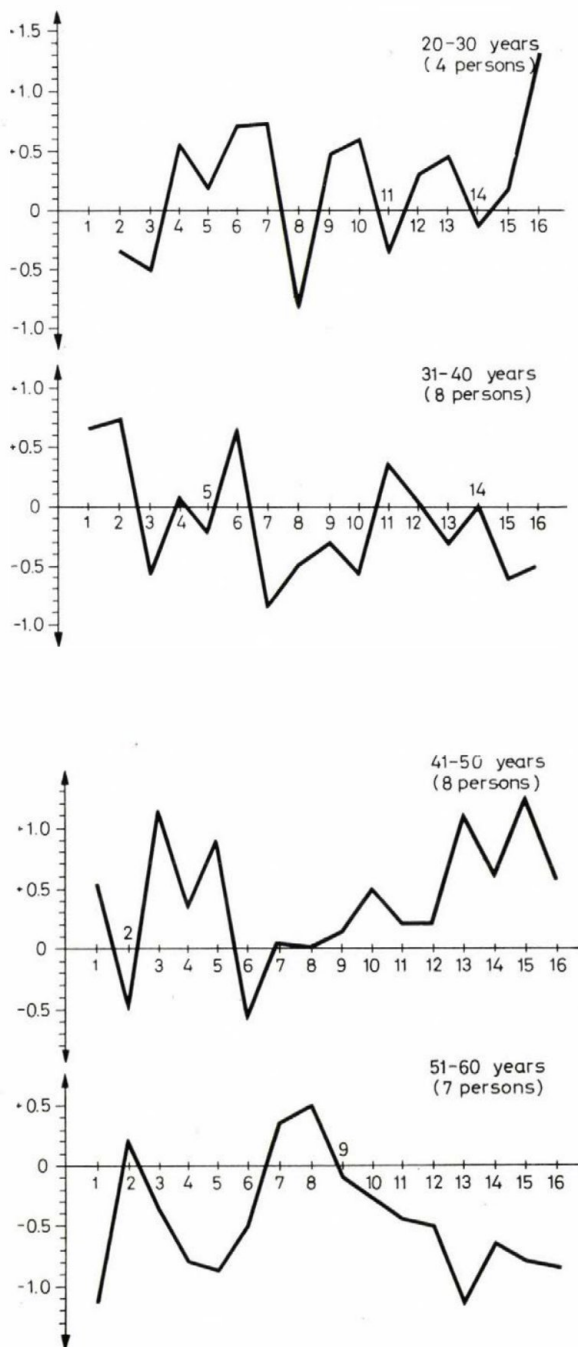


Fig. 2. Difference in average titre according to age groups from the average titres for 27 women. For explanation of numerals, see Fig. 1

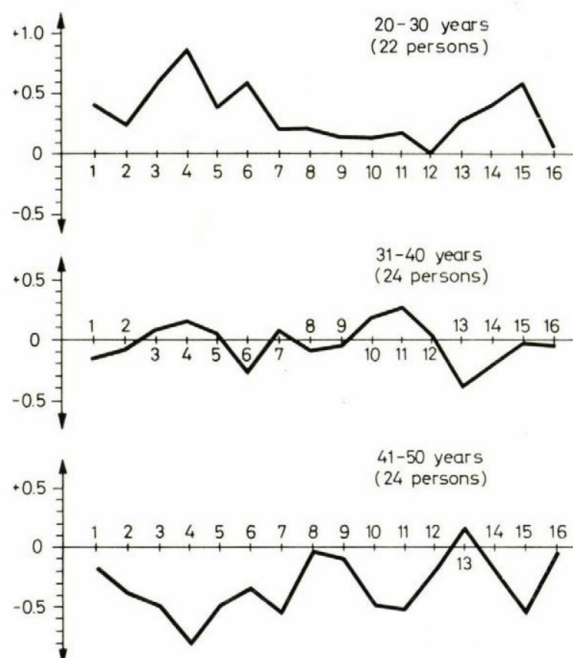


Fig. 3. Difference in average titre according to age groups from the average titres for 73 men. For explanation of numerals, see Fig. 1

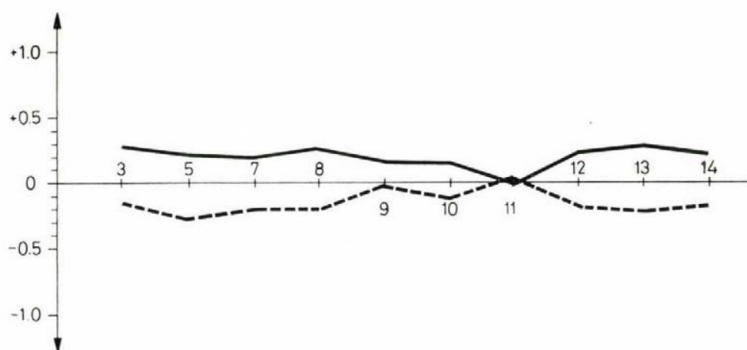


Fig. 4. Difference in average titre according to Rh positive (—, 45 persons) and Rh negative (-----, 55 persons) groups from the average titres for 100 persons. For explanation of numerals, see Fig. 1

Staphylococcal α -antitoxin titres showed no age or sex difference and were, therefore, omitted from Figs 1-3.

Natural antibody titre and Rh factor. Comparison of the average titres of Rh positive and Rh negative persons for 10 different bacterial antibodies is presented in Fig. 4 (both sexes) and in Fig. 5 (females and males separately).

Table V
Sex distribution of bacterial antibodies

Antibody for	Females			Males		
	No.	$\bar{x}$	SD	No.	$\bar{x}$	SD
<i>S. typhi</i> O901	27	4.26	1.68	73	5.08	1.52
<i>S. typhi</i> Vi	27	3.45	1.69	73	4.02	2.03
<i>S. typhi</i> 2	27	4.87	1.64	73	5.13	1.62
<i>S. paratyphi-A</i>	27	4.41	1.32	73	3.89	1.40
<i>S. flexneri</i> 1b	27	5.69	1.46	73	5.21	1.36
<i>S. flexneri</i> 2	27	4.89	1.40	73	4.82	1.40
<i>S. flexneri</i> 3	27	5.20	1.42	73	4.88	1.42
<i>S. flexneri</i> 6	27	6.31	1.85	73	6.14	1.30
<i>S. sonnei</i>	27	5.42	1.83	73	5.47	1.49
<i>E. coli</i> O26	27	5.17	1.33	73	4.24	1.62
<i>E. coli</i> O55	27	3.28	1.51	73	3.40	1.68
<i>E. coli</i> O86	27	6.44	2.09	73	5.52	1.66
<i>E. coli</i> O111	27	4.38	1.77	73	3.50	1.09
<i>P. aeruginosa</i>	27	5.31	1.73	73	4.88	1.71

Table VI
*Sex distribution of staphylococcal
 α -antitoxin titre*

	IU/ml	
	$\bar{x}$	SD
73 men	0.56	0.27
27 women	0.59	0.33
Total	0.56	0.29

Discussion

The studies described in this paper supplement the data of other authors [6, 11] for the natural antibody level in healthy Hungarian adults. The difference between earlier and the present results may be attributed to the fact that different populations were examined (see Fig. 6).

In this study answers were sought to the question whether the natural antibody level was associated with sex, age or with Rh factor. It has been shown that with ageing the incorporation of certain antigens may decrease or the immune response may show an alteration, and that the level of bacterial antibodies is associated with the amount of antigens regularly gaining entrance to the organism [17].

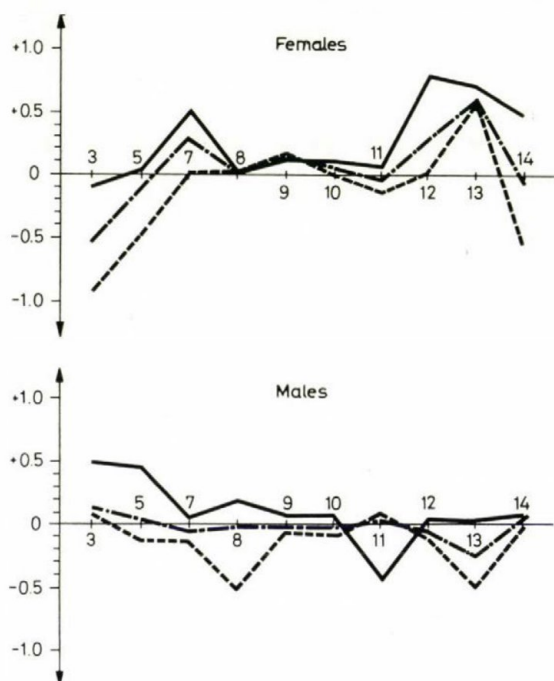


Fig. 5. Difference in average titre according to Rh positive and Rh negative females (upper curve), and Rh positive and Rh negative males (lower curve). — Rh positive, - - - - Rh negative, - . - . total. For explanation of numerals, see Fig. 1

The sex difference in antibody levels may be due to the higher IgM content in women's as compared to men's sera [18]. Although from the present data no conclusions can be drawn as to antibody levels characteristic of sexes and age groups, it may be stated that the same age groups of females and males differed in this respect and the differences were greater among women than among men belonging to various age groups. The antibody titre in men decreased gradually from 20 to 50 years of age, while in women there was no such definite decrease for certain antibodies. The antibody level in Rh negative donors (especially in men) seemed to be lower than in Rh positive persons.

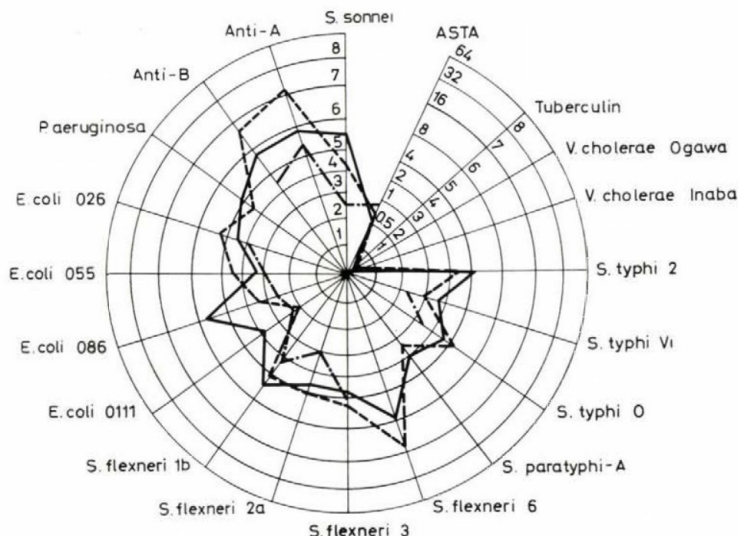


Fig. 6. Antibody titres in different groups of healthy adults in Hungary. — 100 city-dweller blood donors, - - - 103 city-dweller adults, — · — · 300 healthy city-dwellers and country people

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STUDIES ON THE PRESENCE OF ANTIBODIES TO EB VIRUS AND OTHER HERPESVIRUSES IN NORMAL CHILDREN AND IN INFECTIOUS MONONUCLEOSIS

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Summary. Children free from infectious disease have been examined by indirect immunofluorescence for the presence of antibodies to the intracellular capsid antigen of Epstein-Barr virus (EBV). In the first year of life 46%, between 2 and 6 years of age 66%, and between 7 and 14 years 91%, of the children proved positive. The corresponding percentages for the presence of antibodies to cytomegalovirus (CMV) and herpes simplex virus were about 50%, irrespective of the children's age. Serum samples from 69 patients suffering from infectious mononucleosis (IM) were tested for anti-EBV antibodies. Of the 29 Paul-Bunnell-positive patients 22 had antibodies, 11 of them in high titres ($> 1:80$). Of the 40 Paul-Bunnell-negative cases only 21 had antibodies, 8 in high titres. Of the Paul-Bunnell-negative cases, 73% were found to have anti-CMV antibodies, 32% in high titre. The respective percentages for the Paul-Bunnell-positive cases were 42% and 10%.

Since its discovery in 1964 [1] the Epstein-Barr virus (EBV) has become one of the most thoroughly studied viruses. It seems to be related aetiologically to infectious mononucleosis (IM), strictly speaking to the Paul-Bunnell-positive form of IM [2–4]. Furthermore, a close relationship has been found between Burkitt's lymphoma [5, 6] and nasopharyngeal carcinoma [7] on the one hand, and the presence of EBV on the other. In spite of these, little is known of the pathogenicity of the virus.

We have reported on the relationship between EBV and sarcoidosis, and EBV and systemic lupus erythematosus [8, 9]. For a better evaluation of the results, it seemed to be of interest to know the frequency of antibodies to EBV and of those to two further herpesviruses, *viz.*, herpes simplex virus (HSV) and cytomegalovirus (CMV) in Hungarian children.

These questions have been studied in the present work. In addition, serum samples from patients suffering from IM were tested for the presence of antibodies to EBV, and of those to CMV, another member of the Herpesvirus group supposed to be in relationship to IM.

Materials and methods

Sera. Blood samples were collected from children living in the town of Debrecen and its surroundings and admitted to the Department of Paediatrics with various non-infectious diseases. They had not spent enough time in the ward to develop antibodies in response to some infection acquired after admission. From patients with IM, several samples were taken. The sera were kept at -30°C until used.

Demonstration of antibodies. The indirect immunofluorescence (IF) method was used for detecting antibodies to EBV, CMV and HSV. The test for antibodies to EBV was performed in smears of EBV-infected P3HR-1 cell cultures, as described by HENLE *et al.* [3]. The antibodies thus demonstrated are bound to the intracellular capsid antigen. To demonstrate anti-HSV antibodies, HEp-2 cells were infected with the HIL strain of HSV serotype 1. As the cytopathic changes had appeared, the cells were trypsinized, and washed with PBS. The indirect IF test was carried out in smears prepared from these cells. Anti-CMV antibodies were detected in the same manner as the anti-HSV antibodies, except that human embryonic fibroblast cultures infected by CMV were used. Cultures showing extensive cytopathic changes were trypsinized.

The Paul-Bunnell (PB) reaction was performed in the laboratory of László Hospital.

Results

1. *Antibodies in children.* Table I shows the frequency of anti-EBV-positive children in three different age groups. The rate of seropositivity increased from 46% in infancy to 91% at school age. The rate of anti-HSV and anti-CMV seropositivity ranged from 43% to 61%, irrespective of age. Of the 100 children tested, 70, 54 and 50 had demonstrable antibodies to EBV, CMV and HSV, respectively. The majority of anti-EBV seropositives had titres not higher than 1 : 80 (Table II).

Table I

Incidence of antibodies to EBV, CMV and HSV in children

Age	No. of children	No. of seropositive children			Per cent of seropositive children		
		EBV	CMV	HSV	EBV	CMV	HSV
1-12 months	28	13	17	14	46	61	50
2-6 yr	36	24	18	20	66	50	56
7-14 yr	35	32	19	15	91	54	43

Table II

Distribution by antibody titres of 100 children

Antibody	Children with antibody titres					
	< 1 : 10	1 : 10	1 : 40	1 : 80	1 : 160	1 : 320
anti-EBV	30	23	26	18	1	2
anti-CMV	46	18	17	14	4	1
anti-HSV	50	21	11	16	1	1

2. *Infectious mononucleosis.* Two or more blood samples were obtained from each of the 19 cases of IM. The first samples were taken in the acute phase of the illness. The PB test was positive in 8 and negative in 11 of the acute-phase samples.

Of the PB-positive cases (Table III), two had no demonstrable anti-EBV antibody in the acute-phase sample; one of these became positive later, whereas

Table III

*Anti-EBV and anti-CMV titres of paired (or triple)
serum samples from patients with PB-positive IM*

Cases	PB titre	anti-EBV titre	anti-CMV titre	Interval between samplings, days
Gy. Gy.		10	<10	
29 yr	32	40	<10	14
♀		10	<10	35
S. Zs.		<10	<10	
17 yr ♀	32	10	<10	7
M. N.		80	<10	
21 yr ♀	256	160	10	7
S. J.		<10	10	
26 yr ♂	64	<10	40	4
R. L.		10	80	
23 yr ♂	128	160	160	5
Sz. Z.		80	<10	
20 yr ♂	1024	80	<10	35
Sz. K.		80	<10	
18 yr ♀	1024	160	<10	10
D. B.		10	<10	
23 yr ♀	64	40	<10	14

in the serum of the other the anti-CMV titre showed a four-fold rise. Six of the PB-positive patients had acute-phase anti-EBV antibodies and five of these showed increase in titre during convalescence.

In five of the 11 PB-negative cases there was no anti-EBV antibody in the serum samples. One initially anti-EBV-negative case turned into positive during convalescence, and in the remaining five cases an increase in the titre occurred. Anti-CMV titres were present in all the 11 cases. In four subjects including two EBV-negative ones, these antibodies showed a well-defined

Table IV
*Anti-EBV and anti-CMV titres of paired (or triple)
 serum samples from patients with PB negative IM*

Cases	anti-EBV titre	anti-CMV titre	Interval between samplings, days
K. Gy. ♀	<10	10	
17 yr	<10	10	28
K. E. ♀	<10	10	
19 yr	<10	640	13
K. G. ♀	10	80	
31 yr	40	160	10
M. I. ♀	<10	10	
17 yr	<10	10	18
H. R.	10	10	
30 yr	40	40	12
♂	80	640	28
Z. Gy.	<10	40	
26 yr	40	160	5
♂	40	320	20
K. F. ♀	<10	<10	
20 yr	<10	640	12
B. R. ♂	40	640	
33 yr	80	640	27
T. L. ♂	10	160	
21 yr	40	160	10
D. S. ♂	160	10	
18 yr	320	10	7
V. É. ♀	<10	40	
20 yr	<10	80	8

Table V
Anti-EBV titres of PB-positive and PB-negative cases of IM

	Patients with an anti-EBV titre of						
	< 1:10	1:10	1:40	1:80	1:160	1:320	1:640
PB-positive, No. 29 cases	7	3	3	5	7	2	2
per cent	24		38			38	
PB-negative, No. 40 cases	19	6	4	3	4	2	2
per cent	48		28			20	

increase. In five cases there was no demonstrable increase in CMV titre, and in two of these cases the acute-phase titre was already relatively high (Table IV).

At least one serum sample was available from each of 69 cases. These are shown according to anti-EBV titre in Table V. If more than one serum sample was tested, the highest value was taken into consideration. Of the PB-positive cases 24% had no, whereas 38% had high-titre ($>1:80$), anti-EBV antibodies; for the PB-negative cases the respective percentages were 48% and 20%.

Among the PB-negative patients, 27% had no demonstrable antibody to CMV, whereas 32% had high titres ($>1:80$); in the PB-positive cases these percentages were considerably different, *viz.*, 58% and 10%, respectively (Table VI).

Table VI

Anti-CMV titres of PB-positive and PB-negative cases of IM

	Patients with an anti-CMV titre of						
	$< 1:10$	$1:10$	$1:40$	$1:80$	$1:160$	$1:320$	$1:640$
PB-positive, No. 29 cases	17	2	5	2	1	3	—
per cent	58		32			10	
PB-negative, No. 40 cases	11	5	8	3	7	2	4
per cent	27		41			32	

Discussion

The anti-EBV seropositivity rate shows a well-defined rise with age in childhood. In previous studies [8, 9] we found 55–75% seropositivity in adults, against the 91% frequency now obtained for school children. It may be supposed that in some adults the concentration of antibody acquired in childhood falls below the threshold of demonstrability many years after the infection.

It seemed of interest to compare our findings with those obtained in other countries (Table VII). The rate of EBV seropositivity for pre-school children examined by us is lower than those published for pre-school children living under poor social conditions [10–12, 13], but higher than the corresponding rates for children living under high social conditions [18, 19].

The rate of seropositivity to CMV and HSV attained in school age shows no further rise. Essentially similar conclusions were drawn by PORTER *et al.* [13] who used the complement-fixation test.

2. Based on seroepidemiological studies, first of all the PB-positive cases of IM have been supposed to be in an aetiological relationship to EBV

[2-4, 20]. However, PB-positive cases showing neither anti-EBV seroconversion nor a rise in titre have also been observed [21, 22]. In clinically typical PB-negative cases, on the other hand, an aetiological role of the CMV has been made probable by seroepidemiological studies [23]. In some of such cases, an increase in both anti-CMV and anti-EBV titres has been observed [28].

Table VII

*Rates of anti-EBV positivity as demonstrated
in different countries*

Country (State) and reference	Age, year	Seropositivity per cent
Alaska [10]	childhood	100
Uganda [11]	2- 3	89
	14-15	68
Mexico [12]	1- 4	86
Singapore [13]	school age	92
Texas [14]	4- 6	80
	8-14	90
Hungary	2- 6	66
	7-14	91
France [15]	1- 2	48
	11 and above	76-88
North-America (Yale University) [16]	17-18	26
Brazil [17]	9	41
	15-40	88
Sweden [18]	5	43
	15	67
Switzerland [19]	pre-school age	10
	26	48

We found a significant (at least four-fold) rise in the anti-EBV titre in a single case of PB-positive IM. A twofold increase was observed in four cases. In two cases anti-EBV antibodies could not be detected; in these cases, however, convalescent serum was not available. Similar results were obtained by DALENS *et al.* [25] and JONCAS [22].

The frequent absence of anti-CMV antibodies in PB-positive cases of IM, in contrast to the well-defined increase in these antibodies in a considerable proportion of the PB-negative cases, is remarkable. Although anti-EBV antibodies were absent in about half of the PB-negative cases, a slight increase or seroconversion occurred in some of the others. In general, in the PB-positive

cases the anti-EBV, whereas in the PB-negative cases the anti-CMV antibodies tended to be dominant.

The present findings are consistent with the results published by KLEMOLA and KÄÄRIÄINEN [23] and KLEMOLA *et al.* [24], who attributed an aetiological role to CMV in some PB-negative cases of IM. The interpretation of the role of EBV is more complicated. In accordance with other authors [21] we have failed to demonstrate a consistent relationship between EBV and IM. Nevertheless, the increase in anti-EBV titre in a considerable number of cases supports the view of DALENS *et al.* [21] concerning the aetiological role of EBV in certain cases of PB-positive and PB-negative IM. The simultaneous increase in titre of the antibodies to both EBV and CMV in a few cases is suggestive of a double infection. In these cases, a pre-existing dormant infection by one of these viruses might be activated by a primary infection due to the other virus.

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FACTORS INFLUENCING THE STABILITY OF ACID-PRECIPITATED POLYVALENT BORDETELLA PERTUSSIS BULK SUSPENSIONS

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Summary. According to the statistical analysis of the potency values of acid precipitated polyvalent bulk suspensions of *Bordetella pertussis*, the potency of suspensions decreased below 8 IU/30 IOU during storage for 5 to 9.8 years. The average annual decrease over a 13-year period was 2.03 IU/30 IOU. The periodicity in the decrease of potency values was assumed to be connected with changes in the quality of the casein hydrolysate ingredient of the medium. Periodic functions employed to approach potency values with limits of 1 SD falling within the range of 64–156% indicated that the potency decreased below 8 IU/30 IOU after 9 years.

According to the Requirements for Pertussis Vaccine of the World Health Organization, the potency of pertussis and pertussis containing combined vaccines shall not be less than 4 IU in the volume recommended as a single human dose [1]. The potency of *Bordetella pertussis* vaccines, as examined with the active mouse protection test, decreases during the storage. KENDRICK *et al.* [2] showed that some pertussis vaccines may retain their mouse-protective activity for 8–10 years. Other data indicated that during a 4-year observation period the potency did not decrease [3]. At the time of the above investigations the International Standard for Pertussis Vaccine had not yet been established and thus the results of active mouse protection tests were difficult to compare. During storage it is important to estimate the loss of potency of the suspensions, since this is the only way to ensure value of the vaccines. In the present work, potency testing of acid precipitated polyvalent *B. pertussis* bulk suspensions prepared for 13 years was carried out. A statistical analysis of the results allowed a more exact understanding of the time-course of the potency decrease.

Materials and methods

Bacterial strains. The suspensions tested were prepared from the following phase I *B. pertussis* strains: 41 405 (serotype 1, 2, 3), 324E (serotype 1, 2), 358E (serotype 1, 2), CN2894 (serotype 1, 2), CN2896 (serotype 1, 2), CN2897 (serotype 1, 2) and 59 (serotype 1, 3).

Medium and cultivation. Modified Cohen–Wheeler medium containing 0.5% resin was used throughout. During the 13-year period, 17 different batches of casein hydrolysate were employed; their total nitrogen content ranged from 7 to 9%, the alpha nitrogen content from 6.3 to 8.8%, the chloride concentration between 50 and 100 mg/kg. Cultivation was performed at 35°C on a shaker or in a fermentor for 20–24, occasionally for 48 hr [4, 5].

Opacity was determined photometrically by comparison with the International Opacity Reference Preparation [6] and expressed in International Opacity Units (IOU).

Bulk suspensions were prepared by acid precipitation as described by PUSZTAI *et al.* [7]. The suspensions were inactivated at 56°C for 1 hr, preserved with 0.02% sodium ethylmercurithiosalicylate, and stored at 4°C.

Polyvalent bulk suspensions contained at least 5 of the 7 above mentioned *B. pertussis* strains. The term "suspension" hereafter means polyvalent bulk suspension.

Mice. CFLP random-bred mice of both sexes weighing 14–16 g were used.

Potency tests followed closely the method suggested in the Requirements for Pertussis Vaccine of the WHO [1]. As a standard, the Hungarian Standard Pertussis Vaccine containing 51 IU per ampoule was used [8]. For challenge, KENDRICK's *B. pertussis* 18-323 strain was used.

Mouse toxicity testing was performed as described in the WHO Requirements for Pertussis Vaccine [1].

Histamine-sensitizing activity was determined by PRESTON's method [9] as modified by Joó *et al.* [10].

Statistical evaluation. ED₅₀ values were calculated on the basis of the method WORCESTER and WILSON [11]. Analysis of regression was performed by FINNEY's method [12]; periodicity tests were carried out with a type 709 WANG computer.

Results

Between 1960 and 1972, a total of 53 polyvalent *B. pertussis* bulk suspensions (Batch No. 28–80) were produced. The mean of the results of 185 potency tests carried out immediately after preparation was 19.45 IU/30 IOU (1 SD $\pm$ 14.66 IU/30 IOU). Mean opacity of the suspensions was 350 IOU/ml. Potency was expressed in IU/30 IOU, as since 1968 the commercial DTP vaccines used in Hungary contain 30 IOU *B. pertussis* bacteria per ml. (Up to 1968 DTP vaccines contained 45 IOU *B. pertussis* per ml). Immediately after manufacturing, the potency of suspensions met the requirements; the geometric mean of the potency of the least effective suspension (Batch No. 58) was 10.7 IU/30 IOU.

In 1973, each suspension was tested for potency in 2 or 3 experiments. The 132 potency values obtained in this manner were plotted against the time of preparation (the year in which the suspension had been produced) and were analyzed according to four different functions (Fig. 1). The least residual variance was obtained with the exponential, while the highest correlation coefficient with the logarithmic, function. These two curves intersect the straight line representing 8 IU/30 IOU at years 5 and 8.5, respectively. The 53 suspensions had been cultured on Cohen–Wheeler medium prepared with 17 different batches of casein hydrolysate. Fig. 2 shows the analysis of the 132 potency values plotted against the casein hydrolysate ingredient of the medium according to the 4 functions. In this plotting the batch No. of casein hydrolysate used for the preparation of the majority of bulk suspensions pooled in the given polyvalent bulk suspension was taken into account. A re-grouping of potency values according to the batches of casein hydrolysate was considered necessary since this ingredient had a considerable influence on the growth of cultures. On the other hand, only batches ensuring abundant growth were used. Other factors considerably influencing growth, such as

resin, trace elements, etc., remained the same during the period of the study; average opacity was about 45 IOU/ml. The lowest residual variance was obtained with the logarithmic natural, the highest correlation coefficient with the logarithmic, function. The 8 IU/30 IOU straight line is crossed by these

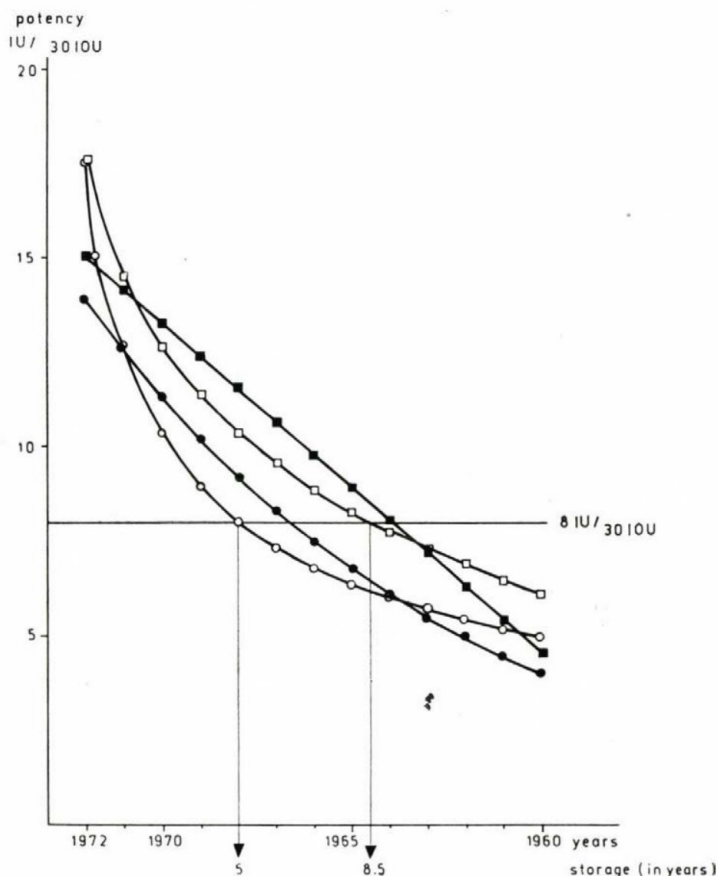


Fig. 1. Regression analysis of 132 potency values plotted against time according to four different functions; $\square-\square y = a_0 + a_1 \lg x$, $\blacksquare-\blacksquare y = a_0 + a_1 x$, $\circ-\circ y = a_0 \cdot x^{a_1}$, $\bullet-\bullet y = a_0 \cdot e^{a_1 x}$.

curves at 5.7 and 9.8 years, respectively. Table I presents the characteristics of these functions.

If the annual mean potency values determined after production are regarded as 100%, the loss of potency in per cents can be characterized by the potency values obtained in 1973 (Fig. 3). In the year following the preparation of the vaccine, the potency increased, then decreased and reached a 50% value after 8 years. The known loss of potency of course does not allow to

forecast the potency of a given suspension at a given point of time. This value can be interpreted only statistically.

The average annual loss of potency can be evaluated for each year. These values, plotted against time, give a logarithmic relationship (Fig. 4). The curve directly indicates the annual average potency decrease after production. This curve allows to estimate the expiry date of any suspension on the basis of the potency determined immediately after preparation.

Table I

*Characteristic data of regression analysis
of 132 potency values in the function
of time and of casein hydrolysate batches*

	Time	Casein hydrolysate
Functions of highest R	$y = a_0 + a_1 \log x$ $R = 0.5417$ $a_1 = -10.3580$ $a_0 = 17.5987$ $S_{xy} = 5.4581$	$y = a_0 + a_1 \log x$ $R = 0.5507$ $a_1 = -11.2470$ $a_0 = 20.6555$ $S_{xy} = 5.7379$
Functions of lowest S_{xy}	$y = a_0 \cdot x^{a_1}$ $R = 0.4688$ $a_1 = -0.4973$ $a_0 = 17.8370$ $S_{xy} = 2.0811$	$y = a_0 \cdot e^{a_1 x}$ $R = 0.4791$ $a_1 = -0.0870$ $a_0 = 18.6038$ $S_{xy} = 2.1153$

R = correlation coefficient

S_{xy} = square root of residual variance

a_1 and a_0 = constant values

The 132 potency values were grouped and averaged according to the year of production. The curve representing the average values shows a decreasing tendency with several rises and falls. To explain the reason for the positive and negative deviations, first the influence of conditions of the potency assays was examined. After excluding the potency values with an SD higher than the allowed 64–156% [1], 76 data remain. If these are plotted, the resulting curve is parallel with the former curve. If potency values obtained in experiments where the challenging dose was lower than 100 LD₅₀ [1] are disregarded, 63 data remain. The curve for these is also similar to the above ones (Fig. 5). Accordingly, conditions for the potency tests were not responsible for the rises and falls in Fig. 5.

In Fig. 6, 76 potency values within the SD limits of 64–156% have been approximated by periodic functions (sinus, cosinus, etc.). The computer was programmed to select the function which comes nearest to the data of intervals of changing trends. The abscissa shows both the time of production and the

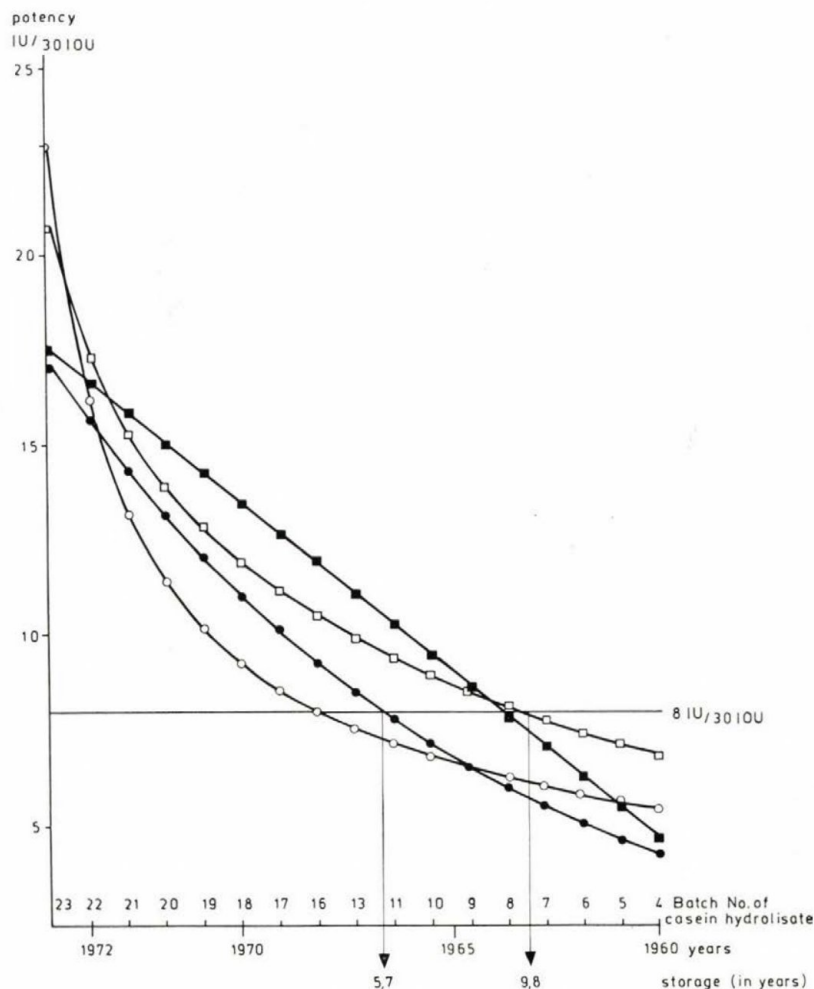


Fig. 2. Regression analysis of 132 potency values plotted against casein hydrolysate batches according to four different functions; $\circ - \circ y = a_0 \cdot x^{a_1}$, $\bullet - \bullet y = a \cdot e^{a_1 x}$, $\square - \square y = a_0 + a_1 \lg x$, $\blacksquare - \blacksquare y = a_0 + a_1 x$

batch of casein hydrolysate used for the culture medium. This potency curve decreased below 8 IU/30 IUU after 9 years.

In Table II, 76 potency values within the SD limits of 64–156% were grouped and averaged according to the year of production of the correspond-

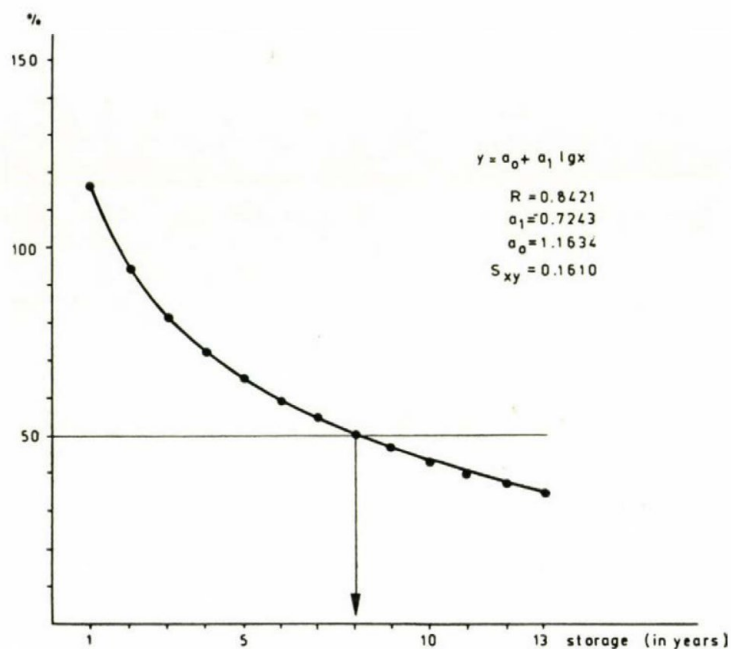


Fig. 3. Potency decrease of 53 *B. pertussis* suspensions in percentage of potency found at the time of production

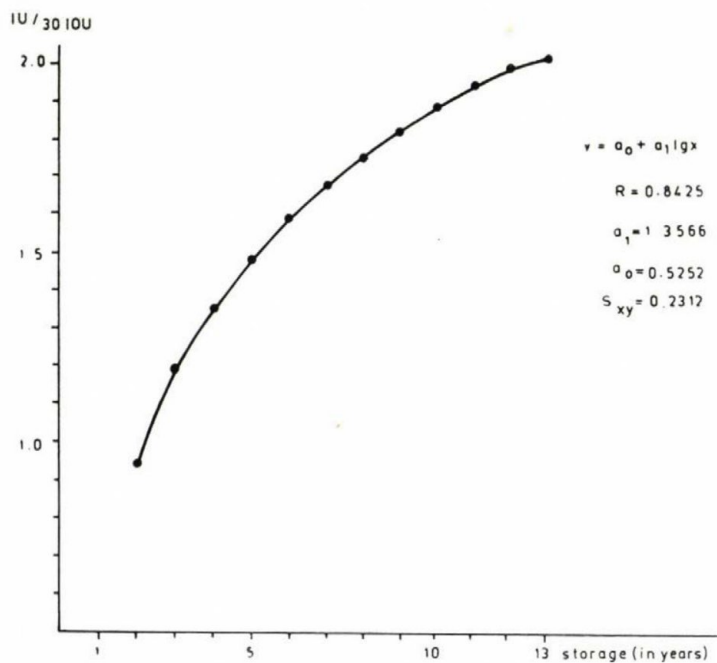


Fig. 4. Expected average annual loss of potency of *B. pertussis* suspensions

ing batches. Then the potency values were rearranged for different batches of casein hydrolysate so that the number of years and the number of casein hydrolysate batches (17) as abscissa values corresponded to each other.

Thus, if one batch of casein hydrolysate had been used for more than one year, potency values of all the suspensions prepared from this batch were

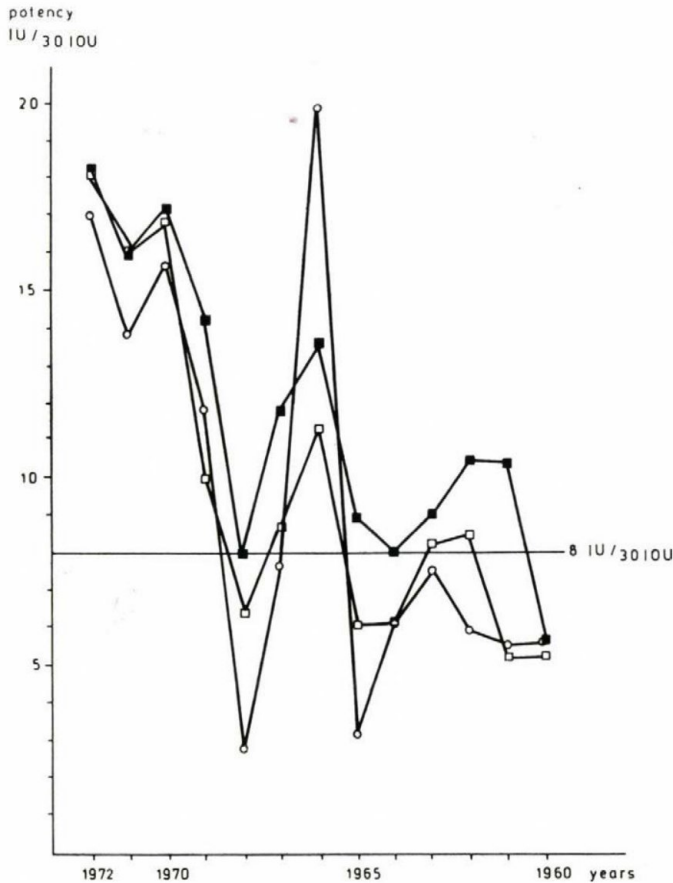


Fig. 5. Effect of the standard deviation of potency test and of the virulence of the challenging organism on the course of potency decrease of *B. pertussis* suspensions. $\square$ — $\square$ mean potency values according to year of production; $\blacksquare$ — $\blacksquare$ mean potency values, with limits of 1 SD less than 64–156% according to year of production; $\circ$ — $\circ$ mean potency values obtained with the challenge dose not less than 100 LD₅₀, according to year of production

considered in every year in the evaluation of the annual average potency. On the other hand, if in a year more than one batch of casein hydrolysate had been employed, the annual average was calculated from the potency values of all the suspensions, prepared from the corresponding batches. These data are presented in Fig. 7. The fluctuations of the loss of potency with time closely

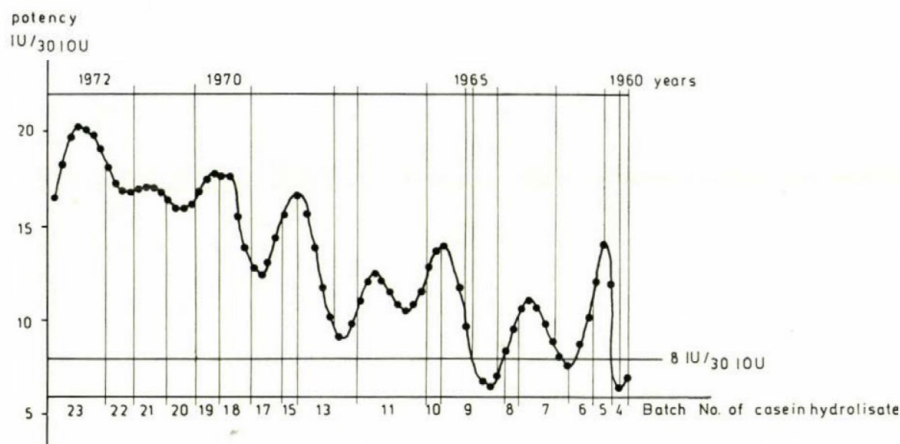


Fig. 6. 76 potency values (limits of 1 SD less than 64–156%) approached by sinus and cosinus functions. The relationship of potency values with time and with casein hydrolysate batches can be read directly

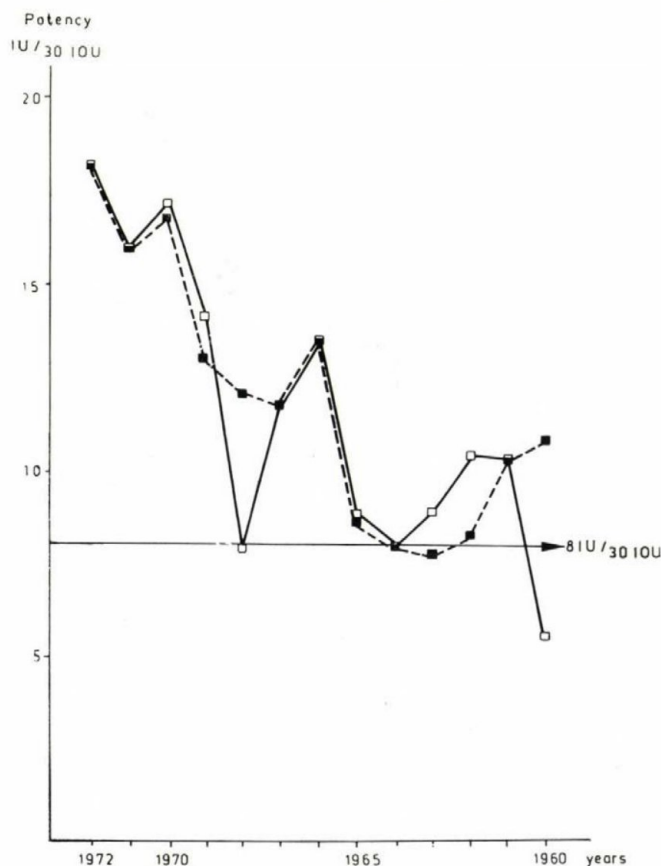


Fig. 7. Mean of 76 potency values according to year of production: (limits of 1 SD less than 64–156%) as a function of time (□—□) and of different casein hydrolysate batches (■—■)

meet the curve of potency values plotted against the casein hydrolysate batches with the only exception of the year 1968. The data in Table II were subjected to periodicity analysis. Mathematically, the periodicity of a process is proven when the residual variance (S_f) of periodic functions (sinus, cosinus) approaching the given data is less than the variance of the data (S_y). The analysis showed that both in the case of the loss of potency plotted against the time of production and of the batch of casein hydrolysate, $S_f < S_y$ ($2.94 < 3.38$ and $1.77 < 3.44$, respectively); which proves the periodicity of the decrease of potency values. The results are set out in Fig. 8. Accordingly, the periodicity of the decrease of the potency values can be explained by differences in the casein hydrolysate batches, *i. e.* in the quality of the medium.

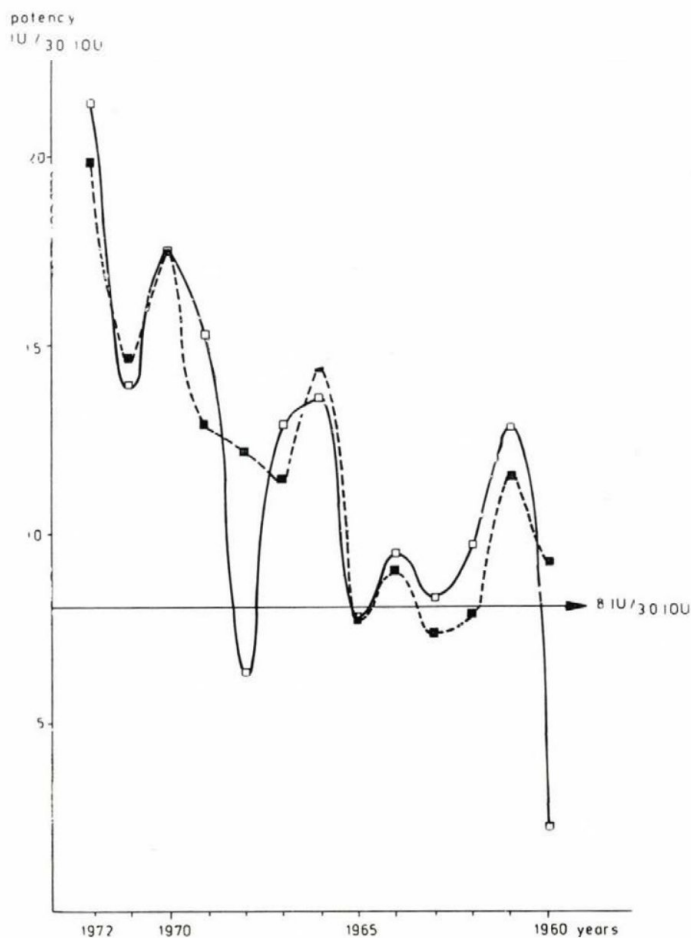


Fig. 8. Periodicity testing of 76 potency values: (limits of 1 SD less than 64–156%) as a function of time (□—□) and of different casein hydrolysate batches (■—■)

Statistical analysis of the opacity of cultures, 52 determinations of histamine-sensitizing activity (HSD_{50}) and 100 toxicity tests indicated that these factors were not associated with the periodicity of potency decrease.

Table II

Mean of 76 potency values (limits of 1 SD less than 64–156%) of 53 polyvalent B. pertussis bulk suspensions according to the year of production in the function of time and of casein hydrolysate batches

Year	Mean of potency IU/30 IOU	Casein hydrolysate Batch No.	Mean of potency IU/30 IOU
1972	18.14	22, 23	18.21
1971	15.93	20, 21	15.88
1970	17.13	18, 19	16.8
1969	14.18	13, 15, 17	13.05
1968	7.9	13	12.08
1967	11.79	11	11.78
1966	13.52	9, 10	13.6
1965	8.9	9	8.71
1964	8.03	8, 9	8.05
1963	8.99	7, 8, 9	7.79
1962	10.49	5, 6, 7	8.29
1961	10.4	4, 5	10.38
1960	5.6	4	10.9

Discussion

The time course of potency decrease of pertussis vaccines has been studied by several authors. According to experiments of the Whooping-cough Immunization Committee of the Medical Research Council, the potency decrease amounts to 30–40% (0.2 logarithmic units) per year [13]. KENDRICK et al. [2] showed no potency decrease in liquid and freeze-dried vaccines in 9 years. This finding, considering the standard deviation of the method, means that the annual decrease did not surpass 1–2%. In our studies the Hungarian Standard Pertussis Vaccine showed no potency decrease during more than 10 years [8]. COHEN, as cited by MAALØE [3], demonstrated in 150 tests with liquid vaccines no decrease in 4 years.

The contradictory data have made us to examine the time course of the potency decrease of our pertussis vaccine. Examination of acid-precipitated

vaccine, in the lack of literary data, seemed especially desirable. During the 13 year observation period, the average annual potency decrease amounted to 10% (0.05 logarithmic units). This finding is about half-way between the seemingly contradictory data of other authors, which may be explained by the fact that the potency decrease in a given year varies with the time elapsed since production of the vaccine. From the logarithmic function reflecting this relationship, the potency decrease can be estimated in anticipation.

Paradoxically, it has been shown that in the first year of production the suspension may exhibit an increasing potency. On the other hand, the toxicity of the suspensions decreases in parallel. Since toxic antigens are diminishing the immunological response, the potency, as the resultant of the two processes, is supposed to increase. In other words, the activity of labile toxic antigens (heat-labile toxin, lymphocytosis-promoting factor) decreases more rapidly during storage than the immunizing potency of protective antigens.

The present studies indicate that the contradictory data described in the literature may be due to the periodicity of potency decrease. Examination of all measurable parameters in our laboratory from potency testing to preparation, has pointed to quality changes in the casein hydrolysate, as a probable factor responsible for the periodic potency decrease. However, as the fall of potency in the year 1968 is not reflected in the curve representing the quality of casein hydrolysate, other, unknown factors may also play a role in the phenomenon.

Acknowledgement. We are indebted to Mr. G. SZABÓ for statistical evaluation.

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EFFECT OF SOME AMINOGLYCOSIDES UPON A DRUG DEPENDENT ESCHERICHIA COLI STRAIN

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Summary. *Escherichia coli* Sd-4-73 drug dependent strain was sensitive to kanamycin, gentamicin and neomycin in the absence of drugs stimulating cell multiplication. This sensitivity was not considerably influenced by streptomycin, while paromomycin afforded protection against both bacteriostatic and bactericidal effects. The paromomycin concentration optimum for protection was different from its concentration optimum for multiplication.

In an earlier paper [1] evidence has been presented that streptomycin (Sm) dependent *Escherichia coli* strain grew on short-chained alcohols in the absence of Sm. On the basis of this finding, GORINI *et al.* [2] discovered a phenomenon which they termed “phenotypic masking”, and also the existence of drug^D strains, whose drug requirement can be satisfied with Sm, paromomycin (Pm) or alcohols. These authors studied the colony forming of the drug^D strain on agar plates containing kanamycin A (Km) (Table I). Later a partial inhibition was described in the case of a Pm+Km combination, which was explained by a competition of Pm and Km [3, 4]. In the case of drug^D strain, the bactericidal effect of Km could only be observed when the inoculum had grown on Sm-containing medium, and this bactericidal effect was weaker than the one observed with *E. coli* B strain [3]. From these results

Table I

Growth of drug^D *E. coli* mutant on Km containing solid media*

Drug in inoculum	Drug in medium				
	Nil	Km	Pm + Km	Sm + Km	EtOH + Km
EtOH	—	—	+	—	—
Sm	±	—	—	—	—
Pm	±	—	+	—	+

* After GORINI *et al.* [2].

and from the Km resistance of the suppressed revertant strains they concluded that the drug^D mutation induced Km resistance.

We now report on results obtained with an *E. coli* strain used previously which proved to be drug^D. We studied the effect of Km and some other aminoglycosides depending on the quality and quantity of drugs employed to satisfy the drug requirement.

Materials and methods

Organisms. (a) *E. coli* Sd-4-73 cys⁻ drug^D strain, obtained from Dr. W. SZYBALSKI had been prepared by DEMEREC and HANSEN [5] from *E. coli* B/r and maintained on nutrient agar containing 50 µg/ml Sm or Pm. From the original Sm-requiring form we obtained the Pm-requiring form by means of subculturing in broth containing 5% methanol.

(b) An Sm^S Pm^S spontaneous revertant was selected from the drug^D strain and an Sm^R Pm^S mutant was isolated with N-methyl-N-nitro-N'-nitroso guanidine treatment of the Sm^S Pm^S revertant. This mutant grows on 1500 µg/ml Sm, and was maintained on agar slant containing 50 µg/ml Sm.

(c) *E. coli* B wild strain.

Drugs. Sm-sulphate (Chinoin), oxytetracycline (Chinoin), chloramphenicol (United Pharmaceutical Works), Km-sulphate (containing 1% kanamycin B, Continental Pharma), puromycin (Reanal). The following drugs were prepared in our Institute: gentamicin C (Gm)-sulphate (composition C 1, 18%; C 2, 40%; C 1a, 32%), Pm-sulphate, neomycin B (Nm)-sulphate, hygromycin B (Hm)-sulphate, neamine, paromamine (Pa) and N-acetyl-Pm. Concentrations of aminoglycoside antibiotics refer to free basis with the exception of Gm and Hm.

Media. The minimal medium contained 0.92% K₂HPO₄; 1.2% KH₂PO₄; 0.1% (NH₄)₂SO₄; 0.01% MgSO₄ · 7H₂O; 25 µg/ml L-cysteine HCl; and 0.5% glucose in distilled water.

Complete medium: 1% beef extract (Oxoid "Lab-Lemco" L30) supplemented with 0.5% peptone and 0.5% NaCl.

Preparation of inoculum. Inoculation from agar slant was performed into 5 ml medium containing 50 µg/ml Sm or Pm or 200 µg/ml neamine. The antibiotic applied was always the same as in the agar slant, except in the case of neamine where we started from Sm-agar. The Sm^R Pm^S revertant and the *E. coli* B inoculum grew without any drug. Incubation was carried out in tubes, at 37°C for 16 hr. The medium of the inoculum was the same as the medium used in the corresponding experiment.

Experimental conditions. Test tubes containing 1.0 ml of the culture were incubated at 37°C without shaking.

Determination of MIC values. The investigated antibiotics were tested in serial twofold dilutions, in the presence and absence of the drugs stimulating multiplication. The tubes were inoculated with 10⁵/ml twice washed cells. MIC values was characterized by the concentration in the first tube which had remained clear after incubation for 2 days.

Determination of bactericidal effect. Twice washed cells (5 · 10⁶/ml) were incubated in the presence of various drugs in minimal medium at 37°C for 30 min, then the effect was stopped by dilution with a ten-fold amount of cold distilled water. Platings were made on complete agar medium containing 6% methanol. The survivor cell count was determined after an incubation for 2 days. On this medium, 100% efficiency of plating was obtained independently of the origin of inocula, without any subsequent decay or protective effect.

Results

In Fig. 1, Km sensitivity of drug^D strain in minimal medium is recorded in dependence on Sm or Pm concentration with respect to the bacteriostatic effect. The MIC value for Km in the absence of Sm or Pm was 4–8 µg/ml and

there was no essential importance whether the inoculum had grown on Sm or Pm. On increasing the concentration of Sm, this basic sensitivity did not change while it decreased parallel with increasing Pm concentrations.

The protective effect of Pm was not directly correlated with its growth-stimulating effect since, as it is obvious from the relative generation times measured in the presence of different Pm concentrations, the strongest protective effect was attained at Pm concentrations higher than those necessary

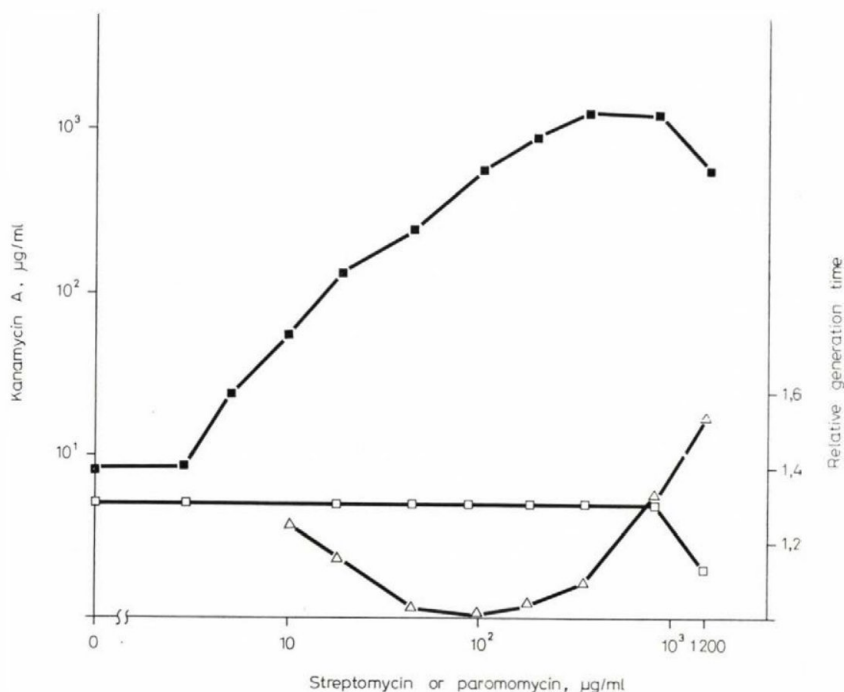


Fig. 1. Dependence of the minimum inhibitory concentration of kanamycin upon the concentration of Sm and Pm in the case of drug^D strain in minimal medium. $\square$ — $\square$ — $\square$ inoculum grown on Sm, dependence on Sm concentration; $\blacksquare$ — $\blacksquare$ — $\blacksquare$ inoculum grown on Pm, dependence on Pm concentration; $\triangle$ — $\triangle$ — $\triangle$ relative generation time as a function of Pm concentration; 1: generation time at 100–200 $\mu\text{g/ml}$ Pm

for the minimum generation time. Eight hundred μg Pm per ml, which partially inhibited growth, displayed an optimum protective effect.

An antagonistic effect nearly similar to that observed on the simultaneous addition of Km and Pm, appeared when 400 $\mu\text{g/ml}$ Pm was added 1 hr after the addition of Km.

The protective effect of Pm and the indifferent behaviour of Sm were detected also in the case of Gm and Nm (Table II). The degree of the protective effect was of the same order of magnitude, being the highest for Km. When

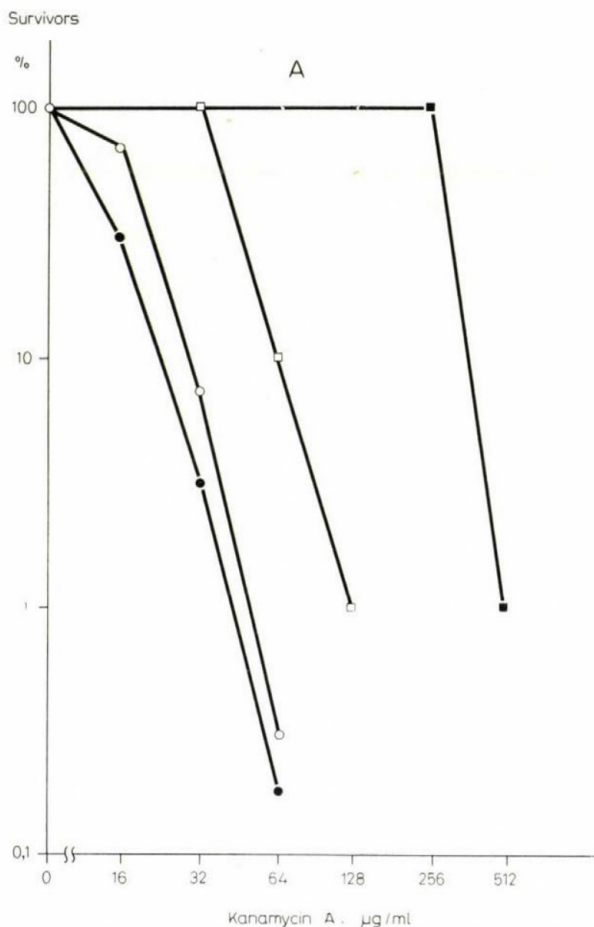


Fig. 2A. Protection against the bactericidal effect of some aminoglycosides in the case of drug^D strain in minimal medium. A: Km, B: Gm, C: Nm, D: Hm; ○—○—○ inoculum grown on Sm, killing in the absence of Pm or Sm; ●—●—● inoculum grown on Sm, killing in the presence of Sm; □—□—□ inoculum grown on Pm, killing in the absence of Pm or Sm; ■—■—■ inoculum grown on Pm, killing in the presence of Pm

Km and Gm were mixed, an additive effect was observed which could also be antagonized by Pm. Pm afforded no specific protection against the effect of Hm. With protein synthesis inhibitors having no aminoglycoside character affecting either 30 S or 50 S ribosome subunit, the resistance observed with Pm was not stronger than with Sm. The above experiments were reproduced qualitatively also in complete medium.

Fig. 2 demonstrates the role of Sm and Pm with regard to the bactericidal effect of Gm, Nm, Km and Hm. In the case of the first three antibiotics, 400 μg/ml Pm was considerably protective, while Sm at the same concentration hardly affected lethality. There was no protective effect in the case of Hm.

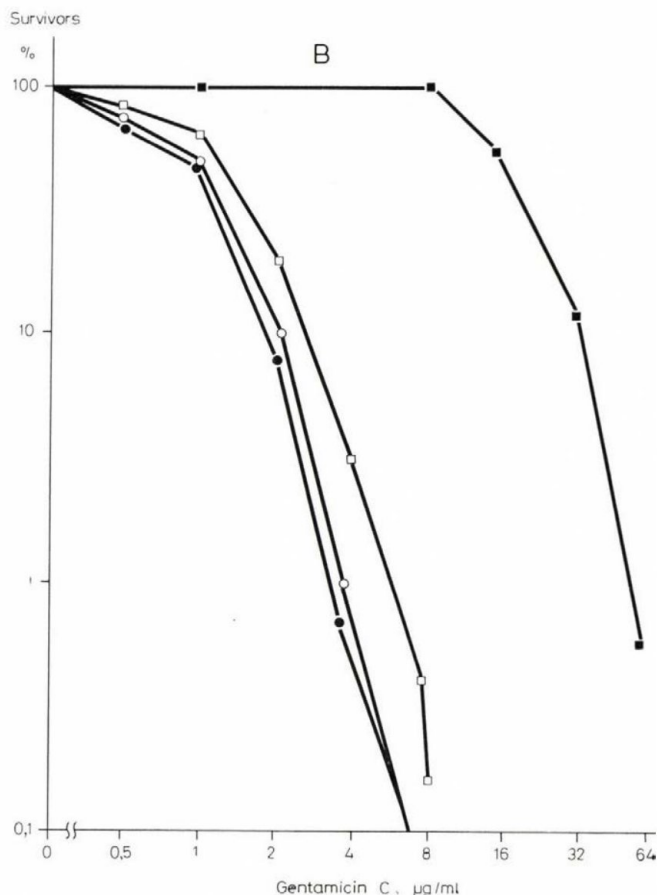


Fig. 2B. Protection against the bactericidal effect of some aminoglycosides in the case of drug^D strain in minimal medium. For point symbols see Fig. 2A

The protective effect of some other substances on which the drug^D strain was able to multiply was tested with inocula grown in Sm or Pm-containing media. In complete or minimal medium containing 6–12% sucrose and 200–1000 µg/ml Pa^x, there was no change in the MIC of Km, Gm and Nm as compared to medium containing Sm. (In sucrose or Pa-containing complete medium growth occurred mostly in the form of bacilli. In minimal medium, filament formation appeared. It could be proved that growth on Pa was not the consequence of some Pm impurity, as growth of drug^D strain in the form of bacilli required 2 µg/ml Pm or 200–300 µg/ml Pa, while for the inhibition of Sm^sPm^s revertant strain on complete medium 0.5–1.0 mcg/ml Pm or 1000 mcg/ml Pa was needed.)

Three per cent ethanol and 5% methanol in complete medium gave also negative results. A weak protection was obtained with 200 µg/ml neamine

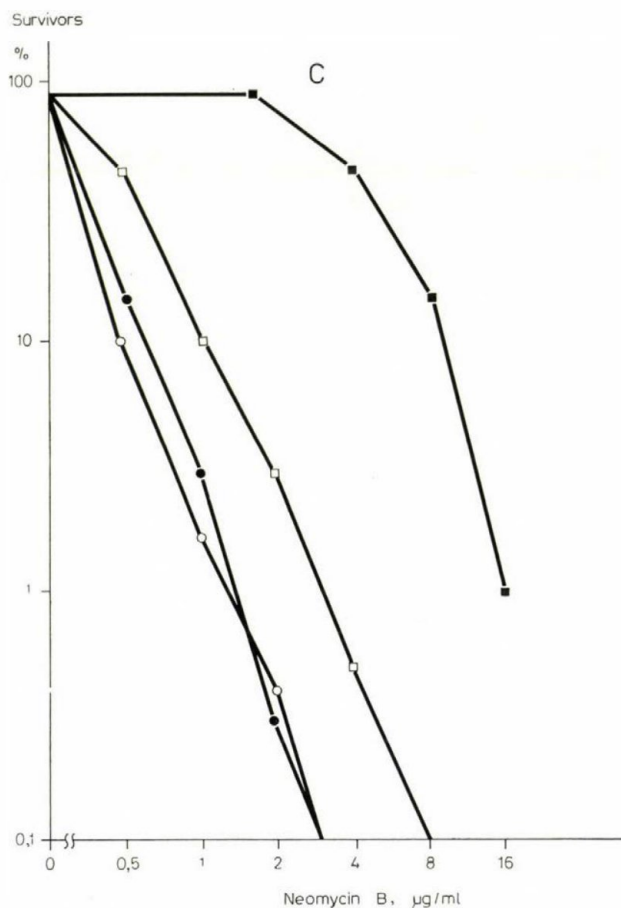


Fig. 2C. Protection against the bactericidal effect of some aminoglycosides in the case of drug^D strain in minimal medium. For point symbols see Fig. 2A

(Table II). The N-acetyl derivative of Pm was ineffective on both multiplication and protection.

Table III displays results obtained with drug^D, Sm^RPm^S revertant and wild *E. coli* B strains. These strains exhibited a roughly similar sensitivity to Km, Gm and Nm. Sensitivity of the revertant strain was not influenced by Sm.

Discussion

GORINI *et al.* [2] and ZIMMERMANN *et al.* [3] tested their drug^D strain to Km sensitivity in the presence of Sm, Pm and ethanol; only a combination of Sm+Km proved toxic and the independent revertant could grow when

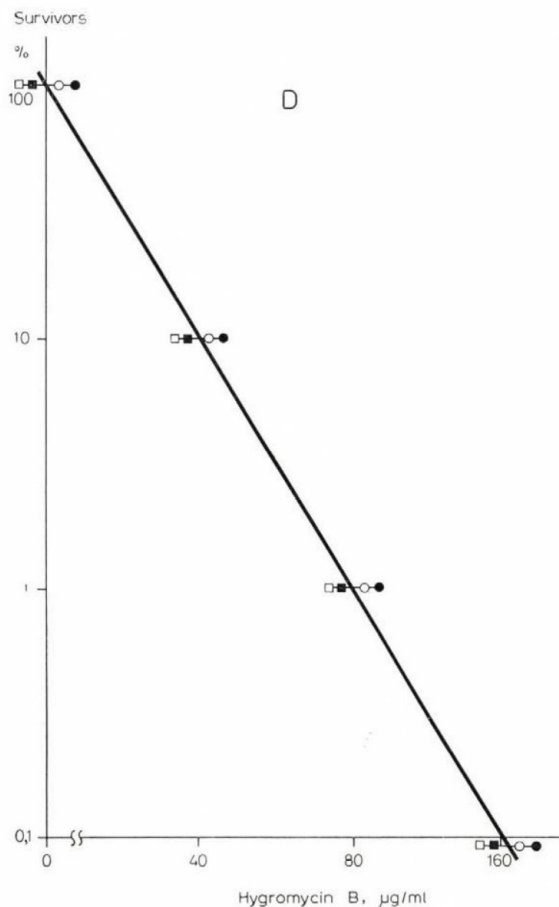


Fig. 2D. Protection against the bactericidal effect of some aminoglycosides in the case of drug^D strain in minimal medium. For point symbols see Fig. 2A

Km was present. They concluded that the mutation to drug dependence induced resistance to Km in comparison to the wild *E. coli* B strain. Moreover, Km made Sm to turn lethal.

QUESNEL *et al.* [6] found that the combinations Sm+Km, Sm+Nm, EtOH+Km and EtOH+Nm were toxic on inocula grown on EtOH. Growth was observed in the presence of Pm+Km, but not in that of Pm+Nm. These authors failed to comment upon GORINI's hypothesis.

Comparison of the sensitivity to Km of the *E. coli* B, the drug^D and Sm^RPm^S strains showed that the drug^D mutation did not unconditionally cause a resistance to Km. Neither was such a resistance observed in the case of our *E. coli* B drug^D strain (unpublished result). Furthermore, our results were indicative of a Pm+Km interaction instead of a Sm+Km one, and the

character of this interaction was also different. Sensitivity to Km was not increased by Sm but it decreased significantly in the presence of Pm.

A similar behaviour was displayed by some related drugs. In the case of Hm, which contains deoxystreptamine and exhibits a misreading effect, Pm had, however, no significant protective effect.

The protective effect of Pm is not closely related to its ribosome-stimulating action, as its concentration optimum for protection is different from that optimum for growth. On the other hand, Pa could replace Pm in enhancing

Table II

Bacteriostatic effect of some antibiotics on the drug^D strain under various conditions

Antibiotics to be antagonized	Drug in inoculum					
	Sm		Pm		Neamine	
	Drug in medium					
	Nil	Sm 400 μg/ml	Nil	Pm 400 μg/ml	Degree of protection	Neamine 200 μg/ml
Kanamycin A	4	4	8	512-1024	64-128	64
Neomycin B	0.5	0.5	1	16	16	1
Gentamicin C	0.5	1	1	32	32	4
Gentamicin C + Kana- mycin A	0.25 + 2	0.25 + 2	0.5 + 4	16 + 256	32- 64	
Hygromycin B	10	20	10	20	2	
Chloramphenicol	1	1	1	1	1	
Erythromycin	64	256	64	256	4	
Oxytetracycline	0.8	1.6	0.8	1.6	2	
Puromycin*	—	16	—	16	—	

MIC values (µg/ml) in minimal medium

* On complete medium, in the presence of maximum 1.6% methanol necessary for dissolving the puromycin. Sm concentrations: 5-100 µg/ml; Pm concentrations: 2-50 µg/ml

multiplication but afforded no protection whatever. According to YAMADA and DAVIES [7], Pa does not cause misreading, while a strong misreading effect was observed by LANDO *et al.* [8].

The growth-stimulating effect of Pa observed in our experiments was in accordance with the results of LANDO *et al* [8]. We have made attempts at separating these two effects by testing the protective effect of N-acetyl Pm, derivative inactive on multiplication; the result was, however, negative. This, of course, does not exclude the possibility of there being a Pm derivative which does not allow growth but exhibits a protective effect.

In contrast to the findings of ZIMMERMANN *et al.* [3], Km as well as Gm and Nm proved not only bacteriostatic but also highly bactericidal on the drug^D strain, irrespective whether the inoculum had been grown on Pm or on Sm. The protective effect of Pm was significant in this respect, too.

With Km, Gm, Nm and Hm, the misreading effect is dependent on the proportion of drug and ribosome concentration [9]. Moreover, a significant resistance could hardly be achieved by a one-step mutation. This indicates that these drugs attach to several sites of the 30 S subunit, in contrast the

Table III

*Kanamycin, gentamicin and neomycin sensitivity of various
E. coli strains in minimal medium free of Sm and Pm*

Effect	Km		Gm		Nm	
	Inhibition*	Killing**	Inhibition	Killing	Inhibition	Killing
Strain drug ^D	4	48	0.5	4	0.5	1.5
Sm ^R Pm ^{S***}	8	64	1	4	2	4
<i>E. coli</i> B	4	16	2	6	4	4

* MIC values in $\mu\text{g/ml}$ after incubation at 37°C for 2 days

** Concentrations in $\mu\text{g/ml}$ causing a 10^{-2} survivor count after incubation at 37°C for 30 min. Plating on 6% methanol-containing complete agar (drug^D strain) or on complete agar without supplement (other strains)

*** Sm had no significant effect up to 400 $\mu\text{g/ml}$.

Inocula were grown with 50 $\mu\text{g/ml}$ Sm (drug^D strain) or without any drug (other strains)

behaviour of Sm and Pm, since here the misreading effect occurred irrespective of the proportion of drug and ribosome, and highly resistant one-step mutants were obtained. There is, however, no direct evidence of the attachment of Pm to a single site. There might exist two separate sites on the ribosome for the binding of Pm and Sm at least in the case of drug^D strains, because otherwise their simultaneous addition would not be toxic [6].

Considering these data, two alternatives offer themselves for explaining the protective effect of Pm. (i) None of the sites of attack of toxic aminoglycosides are identical with that of Pm; the protective effect occurs on the conformational level of some 30 S protein: the toxic substances are not bound or are bound but unable to display their original effect. This would not yet explain our observation that when the concentration of Pm is raised over the optimum for growth (*i.e.* when the Pm site is saturated on every ribosome), the protective effect is further increased. (ii) One of the sites of action of toxic

aminoglycosides is identical with that of Pm, thus it must compete for the critical site. This, however, seems improbable, since the combination of Pm and Sm is toxic, the drug having a misreading effect on being attached to the Pm site should synergize with Sm. Thus, for instance, Sm should increase the toxic effect of Km.

In contradiction with the cited data [9], it could be supposed that Pm has more than a single site of action but at these hypothetical sites Pm can neither increase misreading nor stimulate multiplication of the drug^D strain, yet it can interfere with the effect or the binding to toxic aminoglycosides.

One of the points of action of Pm is probably a site close to the one of Sm, considering the analogous effect of the two drugs on the growth of the drug^D mutant. This site may be on the protein S 12 which has an important role in the binding of Sm [11] but it may also be on other ribosomal proteins or on the 16 S RNA, since this function of S 12 may be an indirect one [10, 12].

The other sites of the protective action of Pm are assumed to be elsewhere than on S 12 protein since this did not seem to be the primary target of the aminoglycosides antagonized by Pm in our experiments.

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DERMATOPHYTES: CONIDIUM-ONTOGENY AND CLASSIFICATION

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Summary. The ontogeny of the conidia of representative *Dermatophyte* species has been examined by time-lapse microphotography. The results are summarized in five main conclusions. (i) the genera *Microsporum*, *Trichophyton* and *Epidermophyton* have holoblastic conidium-ontogeny; (ii) the investigated species exhibit polymeristematic development; (iii) delivery of the conidia occurs by means of a special detaching mechanism: consisting in autolysis of a detaching-cell or cells; (iv) the macroconidia have a primary septum; (v) chlamydospores including "gemmae" and "persistent-organs", strikingly similar to the macro- and microconidia as investigated in aqueous preparations, are also formed. The author classifies the dermatophytes into section III of the system of HUGHES and into the group III/C of TUBAKI's system.

The past 20 years may be considered a renaissance of dermatomycology, when modern microbiological methods and results as well as modern taxonomic and physiological views have gained ground.

The paper of VANBREUSEGHEM [1] dealing with the "hair-bait" method, was a turning point in the research of dermatophytes. This method of mixing soil with keratinous materials, known also as the To-Ka-Va method, is widely used in medical mycology and has made it possible to describe numerous new species of dermatophytes and their perfect forms.

It is a permanent endeavour in mycology to recognize the sexual reproduction of different fungi — first of all those of human importance — in order to find their adequate place in the phyletic system. This fact, however, does not influence the need of recognizing the fungus species in their imperfect, conidial stage as well, the more so, as the perfect forms of most anthropophilic species are not known. Moreover, determination of perfect forms requires special media as well as time-consuming investigations. Finally, the close interspecies similarity of perfect forms in the *Nannizzia* and *Arthroderma* genera makes differential diagnosis difficult, this being based on perfect forms. They are nevertheless important on account of their heterothallism, as the formation of the sexual stage verifies genetically a belonging to the same species in those cases when morphological, pathological and physiological methods fail to yield unequivocal results.

In the past decades it has also become evident that clinical signs provide a poor basis for the identification of the pathogenic agent. Thus, the morpho-

logical and physiological description of fungal species should be completed by indicating the diseases caused by them. It is also worth mentioning that for the identification of dermatophytes cultivated from soil, only the microbiological methods are reliable. Furthermore, owing to the rapid development of the methods, many new features and even numerous new species of dermatophytes have been described. All these have made it necessary to reinterpret the former systematization or to replace it by a new one.

In earlier works [2-4] we have divided the markers of taxa into two groups, *viz.* systematically valuable markers and accessory ones. Since the differentiation of taxa is based on systematically valuable markers, the accessory peculiarities have lost their importance, and thus the characterization of categories has been simplified.

The most important element among the systematically valued peculiarities is the macroconidium. We attach a great importance to them in spite of the fact that this aspect has not been emphasized in medical mycology.

In the past years this rigorous morphological aspect has been replaced by emphasizing the ontogeny of conidia. Classification is now based on ontogeny instead of micromorphological peculiarities. Thus, the character of the conidiogenous cells and, also, the character of conidium development are of decisive importance in classification.

In the present work, we have made an attempt to approach some questions of the classification of dermatophytes on the basis of conidium-ontogeny.

HUGHES [5] was the first to investigate *Deuteromycotina* (*Deuteromycetes*, *Fungi Imperfecti*) in this respect. He was followed by SUBRAMANIAN [6, 7] TUBAKI [8], and BARRON [9], who published a similar conception with some modifications.

Materials and methods

Medium. Sabouraud-glucose-agar microcultures were used in the investigations [10]. *Fungi.* *Microsporium gypseum* F 18, F 128, F 137, Gabon 1, Gabon 6; *M. felineum* (syn.: *M. canis*) OBNI 2; *M. fulvum* F 9, F 26; *M. audouinii* OBNI 1972/1, OBNI 1972/2, OBNI 1972/3; *M. nanum* KÖJÁL 1; *M. vanbreuseghemii* KÖJÁL 1; *M. umbonatum* (syn.: *M. distortum*) KÖJÁL 1; *M. cookei* KÖJÁL 1; *Trichophyton mentagrophytes* OKI 1972/1, OKI 1972/2, OKI 1972/3, Szolnok 1972/1, Szolnok 1972/2; *T. rubrum* OBNI 1972/1, OBNI 1972/2, OBNI 1972/3; *T. fluviumuniense* OBNI 1973/1; *T. vanbreuseghemii* OKI 1; *T. terrestre* F 8, F 22, F 25, F 51; *T. quinckeanum* 1973/1; *Epidermophyton floccosum* OBNI 1971/1, Szolnok 1972/1, Szolnok 1972/2, Szolnok 1972/3, Szolnok 1972/4; *E. ajelloi* (syn.: *Keratinomyces ajelloi* Vanbreuseghem 1952; *Trichophyton ajelloi* Ajello 1968) F 2, F 6, Gabon 2, OKI 1972/1.

Photographs were made traditionally and by time-lapse microphotography.

Results

Results are demonstrated in serial pictures arranged on plates. The first picture in a row usually shows the macroconidium-initial in an advanced stage,

first of all because in earlier phases these are more sensitive to the light and heat effects accompanying exposure. Furthermore, the conidia of dermatophytes, develop exclusively on conidiophores, emerging from the medium, thus being prone to drying and consequently, to lose their characteristic structure. Photographing is made difficult by the fact that the whole supporting structure keeps swaying and changing its plane. To achieve the present quality of pictures we had to eliminate the heat effect and to apply an exposure-time as short as possible.

The speed of development of the macroconidia is not constant; in some phases it may be rapid, requiring a frequency of exposures higher than that applied by us. When we increased the frequency, the organs stopped developing irreversibly, or a long period in darkness was necessary to restart their growth. Usually, the walls of the organs staved in under the effect of one or two exposures, and later the whole organ dried up. Sometimes an incubation for several days resulted in no appreciable development, then a new exposure made after an interval of two or three hours in the dark revealed a series of changes. In some cases, the first photograph is of worse quality than the others because of the short exposure times in the initial, *i.e.* sensitive, stage of development.

Most photographs show the organs in microcultures, in the native state, except when certain peculiarities were studied which require specially treated (*e.g.* aqueous) preparations.

Explanation of plates. In all the explanations 0 hr indicates the time of taking the first photograph which took place several hours after onset on the development of conidium initials.

Plate I, Picture 1 (0 hr) shows the macroconidium (mc) — initial of *M. gypseum*, with a protoplasm drop on the apex. At 1 hr a minimal longitudinal and transversal growth (picture 2), at 4 hr (picture 3) a definite longitudinal growth and thickening are observable. The septum delimiting the terminal cell is clearly visible. In picture 4 (at 7 hr), budding of the collateral mc and a distinct longitudinal growth and swelling are already seen. Such a longitudinal growth may and may not occur simultaneously with the swelling. Picture 5 was taken at 15 hr, when the collateral mc had reached its final length, but showed little if any thickening. Picture 6 was taken at the same time, at another micrometer-position, in order to examine the older, fully developed mc (the two macroconidia lie in different planes). In picture 7 (at 17 hr) a definite swelling is observable on the collateral mc, with the basal part in focus. Picture 8 was taken at the same time, with the apical part in focus. Two parallel photographs (pictures 9 and 10) were taken at 19 hr to show the basal and apical regions. The last picture shows the distinct swelling of the collateral mc. Thus, in this case — in contrast to the behaviour of the main mc — longitudinal development had preceded the transversal one. The orig-

inal magnification (o.m.) was $\times 51$; total observation period: 21 hr; time intervals in hr: 1, 3, 3, 8, 2, 2 and 2.

Plate II. An mc developed on the lateral conidiophore of *M. gypseum*. The septum, next to the starting point, on the base of the conidiophore originating rectangularly from the hypha is clearly visible. The conidiophore consists of one cell the apical third of which begins to swell evenly. A swelling of such a degree takes 2 to 3 hr. In the following four-day period (pictures 2–3) no remarkable change occurred except that the hypha growing from the thallus beside the conidiophore (picture 5) produced vegetative anastomoses (hyphal fusions) with the conidiophore. Eight hr later (picture 6, i.e. at 104 hr) a secondary mc developed from the apical region of the mc-initial, which had stagnated for some days (light-effect!). Considering that the primary mc-initial had remained in an unchanged state for several days, the microculture was stored in the dark for 6 hr, when completely mature secondary mc appeared, with a fully developed detaching-cell (for its interpretation, see below), with protoplasm-release on the detaching-cell, a thinning primary mc, which suddenly has a complete detaching-cell, too (picture 7 at 110 hr). In picture 8 (111 hr) the protoplasm-release becomes larger, at the same time the septum delimited from its detaching-cell disappears. In picture 9 (116 hr) the wall of the detaching-cell of the secondary mc regularly deflates, the protoplasm of the primary mc disappears, and signs of absorption appear on its wall. The whole growth-complex collapses. The last picture (No. 10, 126 hr) shows that the emptying of primary mc and the absorption of its wall continues and thus, the mc is transformed practically into a second detaching-cell (o.m. $\times 51$). Total observation time: 126 hr; intervals: 24, 24, 24, 24, 8, 6, 1, 5, 10 hr.

Plate III. The first 8 pictures show the development of the mc of *M. fulvum*. In pictures 1 and 2, an increase of the distance between the two warts is seen. The warts appear soon and are lying near the basis of the mc. This phenomenon, and the fact that this series shows an apical growth as well as a successive swelling (i.e. transversal growth), suggest that development is in progress simultaneously on different points of the corpus of mc. In picture 3 the developed detaching-cell, in picture 5 a distinct primary septum (see in Discussion) can be seen. An intensive development of the verrucae begins at 12 to 14 (pictures 7 and 8, respectively) after taking of photo 1. It should be noted that photo 1 shows developed mc-initial. The mc of *M. fulvum*, starting from the last cell of the conidiophore, needs approximately 2–4 hr to reach such an advanced state (o. m. $\times 100$). Total observation time: 14 hr; intervals in hr: 2, 2, 2, 2, 2, 2, 2.

Pictures 9–13 show a stubby mc of *M. fulvum* characteristically separated from the conidiophore. The septum separating the apical cell of the conidiophore, the shape of mc, the development of the detaching-cell (picture 12), and the final form of the mc with its verrucose surface and with the destroying

detaching-cell are well visible (o. m. $\times 100$). Total observation time: 30 hr; intervals in hr: 2, 4, 6, 18.

Plate IV. The upper row shows a mc of *M. audouinii*. Picture 2: after the first 3 hr both longitudinal and transversal growth is noticeable. The arrows point to a distinct apical growth. Picture 4: the detaching-cell is delimiting itself (51 hr). Picture 5: during the 73-hr interval, only 1–2 warts and the primary septum appeared and the wall of the detaching-cell began to autolyse (o. m. $\times 100$). Total observation time: 124 hr; intervals in hr: 3, 24, 24, 73.

The lower row represents the mc of *M. nanum*. The arrows point to a wart developed early, the primary septum, and early distinct development of the detaching-cell. In picture 9 absorption of the protoplasm of a detaching-cell is shown (o. m. $\times 100$). Total observation time, 26 hr; intervals in hr: 4, 18, 4.

Picture 10: High magnification (o. m. $\times 765$) shows a small mc of *M. nanum* characteristic in shape. It is divided into two parts by a septum, and displays a short detaching-cell (arrow).

Plate V. The upper row of pictures demonstrates the mc of *Trichophyton rubrum*. As indicated by triangles in pictures 1 and 2, the upper part of the mc corpus is poorly developed in comparison to its apical part. Transversal growth is also seen. Arrows in the same pictures show the gradual swelling of a cell (marked by *a*) lying on the basis of mc, bordered by two septa. In picture 5 protoplasm-release begins from the cell, under the apical cells (cell?), and continues to increase. In association to this, the cell wall deflates. During the 16-hr interval between pictures 7 and 8, important changes took place. Cell *a* splits and an outflow of protoplasm begins along the full length of mc except in the cells of the apical part. The mc breaks in an angle of approx. 180° at *a*. During the next 48 hr, the wall of the emptied part of mc dries up, and its autolysis begins. The completely closed apical region full of protoplasm serves as the persisting organ (o. m. $\times 100$). Total observation time: 96 hr; intervals in hr: 4, 2, 4, 14, 4, 4, 16, 2, 3, 19, 24.

Pictures 13–16 demonstrate the separation of the mc of *T. mentagrophytes*. The detaching-cell appears in picture 14 taken at 24 hr. Five hr later, the detaching-cell begins to autolyse and, after another 5 hr, disintegration of the wall of the detaching-cell is almost complete (o. m. $\times 100$).

In pictures 17–20, the type of separation of different macroconidia of *T. rubrum* is seen when the phenomenon is accompanied by a significant protoplasm-release (o. m. $\times 100$). This process needs thorough investigation and interpretation.

Plate VI. The ontogeny of the conidia of *T. terrestre* can be observed from the beginning to the end in 6 photographs. Photos 3 and 3/a were made simultaneously, so that the conidia of fertile hypha lying in different planes

could be observed close to each other, in the same stage of development. Picture 1: conidiophores (one pointing upwards, the other downwards) on the fertile hypha are visible. Budding of the third conidiophore and formation of an apical conidium are discernible. Picture 2: the formations visible in picture 1 show significant growth; the apical conidium-initial and the conidiophore just beginning to grow in photo 1, are also clearly observable. Two new conidiophores have appeared. Pictures 3 and 3/a: the conidia assume a typical shape, and they are delimited from the conidiophore by a septum. Pictures 4 and 5: ripe conidia with partly dried up supporting structure. The two drawings show the direction of deviation of conidiophores (o.m. $\times 100$). Total observation time: 30 hr; intervals in hr: 7, 13, 10.

Plate VII. Conidium development of *T. terrestre* is somewhat different from the general pattern in pathogenic dermatophytes (see Discussion). However, some of its peculiarities are typical and have a general validity. This is illustrated in a low power picture (o.m. $\times 100$) demonstrating a large area of a microculture. In the left upper corner, the organ-pipe-like arrangement of conidia illustrates the most important peculiarity of this species, the transitional forms between "microconidia" and "macroconidia". It is for this reason that "conidia" are only mentioned with respect to this species (o.m. $\times 252$). The picture marked by x shows that the conidium separates like a budding cell, when no residue of the supporting structure can be detected (o.m. $\times 567$).

Plate VIII. Microconidia and "gemmae" of *Trichophyton* and *Microsporum* genera. Numerous cells of the conidiophore and of the supporting structure autolyze with a simultaneous development of mc. Picture 4 demonstrates a typical detaching-cell occurring at the microconidium. Picture 6 represents a microconidium situated laterally but having a detaching-cell. Pictures 7-10 demonstrate parts of the hypha called "gemmae", simulating microconidia and macroconidia. In pictures 11 and 13 microconidia, attached to or detached from, the conidiophores are seen. Absorption of some conidiophores is observable, while most of the supporting structure remains practically intact (o.m. $\times 100$). Picture 12 shows a regular collar attached to the microconidia, removed mechanically from the wall of the conidiophore (o.m. $\times 567$).

Plate IX. Ontogeny of the mc of *E. floccosum*. The first five pictures represent a complex process. The events visible in the photographs progressed quickly and in parallel. Section marked *a* has lengthened considerably, while section *b* has become an intercalary gemma, an mc (it has become swollen and a septum has appeared in it). In the region of section *a* a lateral mc develops while an approximately spherical growth appears on the apex of the terminal mc. Pictures 3 and 4 were taken simultaneously, showing the intercalary gemma and the mc, respectively. The arrow points to the thinned bridge between this growth and the terminal mc. In picture 4, the above mentioned lateral mc is seen in its fully developed stage. Picture 5 demonstrates initial autolysis

of the detaching-cell of the lateral mc developed in area *a*; the development of the septa of mc; septation of the terminal mc and of its growth (o.m. $\times 100$). Sections *a*, *b* and *c* demonstrate the longitudinal and transversal growth of different areas simultaneously. The four drawings serve the better understanding of changes. Total observation time: 34 hr; intervals in hr: 22, 6, 6.

Pictures 6–9 show the development of a lateral mc. The arrow calls attention to the formation of a septum (o.m. $\times 100$). Total observation time: 36 hr; intervals in hr: 6, 6, 24.

Plate X. The development of the macroconidia of *E. floccosum* is shown in the first five pictures. Arrows point to the same site of mc in pictures 1 and 2; demonstrating that the lower left mc situated on the fertile hypha has markedly grown apically, in addition to its well-defined transversal development. The septum developing in the apical part of the mc situated higher up is also marked by an arrow. The protoplasm-release of the central cell of the terminal mc is well visible. Pictures 4 and 5 illustrate a bulge and also a septum developing on the basal part of the lower left mc. The bulge did not change its position within 24 hr. In spite of this, a septum has appeared in the mc between the bulge and the fertile hypha. Early autolysis with protoplasm-release of the terminal and upper left macroconidia are also shown in picture 5 (o.m. $\times 100$). Total observation time: 60 hr; intervals in hr: 12, 7, 17, 24.

Picture 6 illustrates a well-developed young mc. In pictures 7 and 8 a swelling, a minimum longitudinal growth and a septation of mc are shown. On the basal part, hardly any change can be observed (o.m. $\times 100$). Total observation time: 48 hr.

Pictures 9 and 10 present apical budding and breaking in two of the mc (o.m. $\times 100$ and $\times 252$, respectively).

Plate XI. Different types of terminal macroconidia of *E. floccosum*. The lateral mc frequently budding at the starting point of the terminal mc, and a considerable swelling of its apical cell (cells) is seen. Picture 13 illustrates a secondary mc. Pictures 16–22 demonstrate a detaching-cell under absorption (o.m. $\times 100$).

Plate XII. In some cases, septa can already be observed in an early stage of development in the lateral macroconidia of *E. floccosum*. Septa may be of basal (arrow in picture 11), apical (arrow in picture 9) or irregular arrangement. In pictures 13–18 no septa are seen in the macroconidia. Pictures 17 and 18 demonstrate the characteristic detachment of the lateral macroconidia: swollen hypha residues branch antler-like from the two sides of their basal part, and their liberation from the whole supporting structure is due to an autolysis of cells lying farther from the base of the mc (o.m. $\times 100$).

Plate XIII. Inner structure of the mc of *E. floccosum* in aqueous preparations. Pictures 1–6: macroconidia in various degrees of development. Pictures 7–12: macroconidia in different developmental stages. Picture 13 il-

illustrates a mc detached from the hypha. The mc-initial is shown in picture 1 in profile, in picture 13 in face. The young mc seen in picture 14 rises from the side of the hypha facing us. Pictures 15–17 show well-developed, unseptate macroconidia, detached from the hypha. In picture 18 an mc consisting of a thin basal tube and of a swollen apical cell is shown. In picture 19 an apical septum can only be seen. Pictures 20 and 21 show distinct septation, and picture 22 illustrates septate as well as unseptate macroconidia arising from the terminal cell of a conidiophore. In the two illustrations in the centre of the plate a well-developed mc having no septum as well as an undeveloped mc with septum are demonstrated (o.m. $\times 252$).

Plate XIV. Ontogeny of the mc of *E. ajelloi*. In pictures 2–4 it is shown that the base of an mc or of a conidiophore (in this initial stage it is impossible to differentiate them) is on the side of the fertile hypha facing us. This swollen area emerges slowly but evenly together with a gradual growth of the mc-initial until it can be observed on the upper side of the hypha (see the drawings as well). Pictures 6 and 7 illustrate detaching-cells as well as their autolysis similar to those encountered in the case of previous species (o.m. $\times 50$). Total observation time: 27 hr; intervals in hr: 2, 3, 4, 8, 6, 4.

Picture 8 shows macroconidia in microcultures in their natural arrangement. Picture 9: it is characteristic of *E. ajelloi* that some of its macroconidia stop developing in an initial stage (these were at first thought to be microconidia). Picture 10: a characteristic, regular multiseptate mc, similar to the mc of *M. gypseum* with a well-visible detaching-cell. Picture 11: mature, characteristically arranged lateral macroconidia and the autolysis of parts of the fertile hypha (o.m. $\times 100$).

Plate XV. Development of the lateral macroconidia of *E. ajelloi*. It is characteristic of this species that the laterally branched conidiophores (and conidia) are growing out of the fertile hypha rectangularly. In pictures 1 and 2 the arrows point to the apical growth and the thickening developed in the course of 6 hr. The mc directed upwards must have developed further during this period, while the mc directed downwards did not. Picture 3: another 4 hr later the mc had reached its final size by means of apical growth and swelling. Picture 4: the detaching-cell develops. Picture 5: the detaching-cell autolyzes. Pictures 6–8 illustrate the former area from another aspect: all the points of the total surface of mc (marked by arrows), as well as the apex takes part in longitudinal and transversal growth (o.m. $\times 100$). Total observation time: 29 hr; intervals in hr: 6, 2, 12, 7.

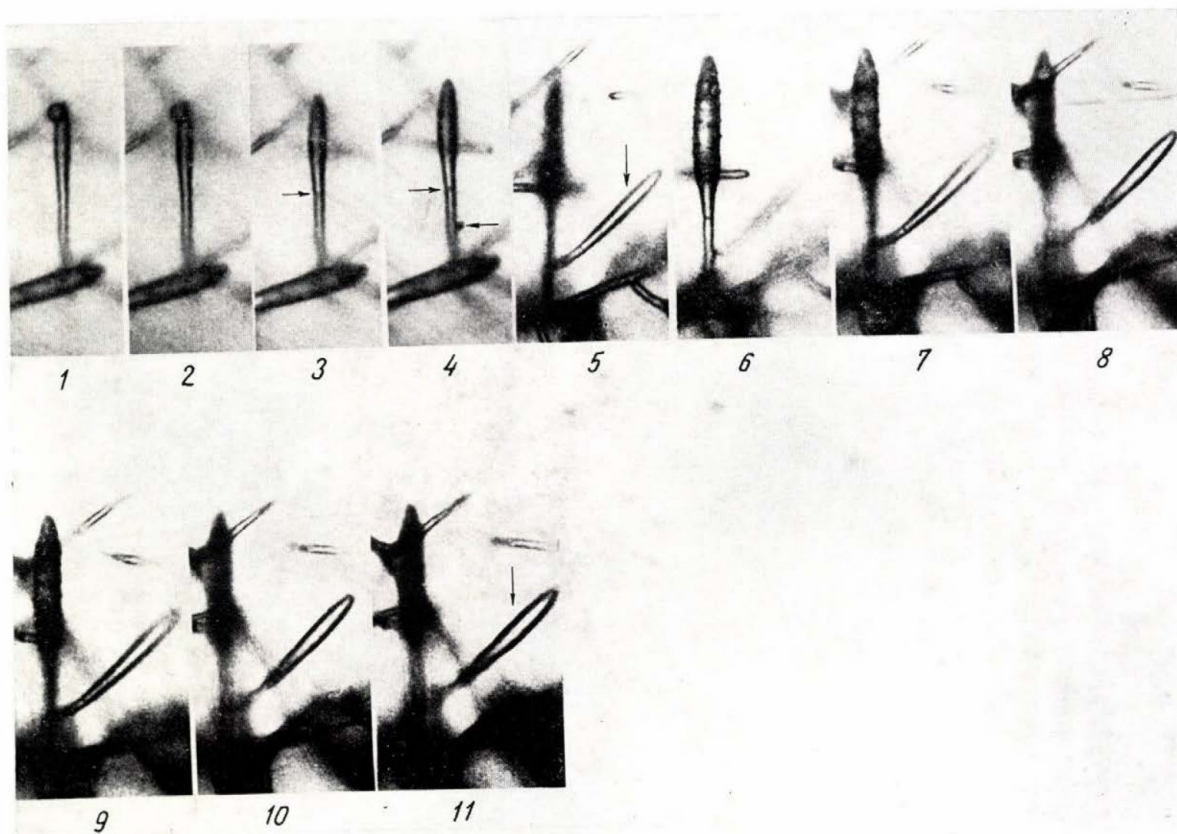


Plate I. Development of a macroconidium of *M. gypseum*

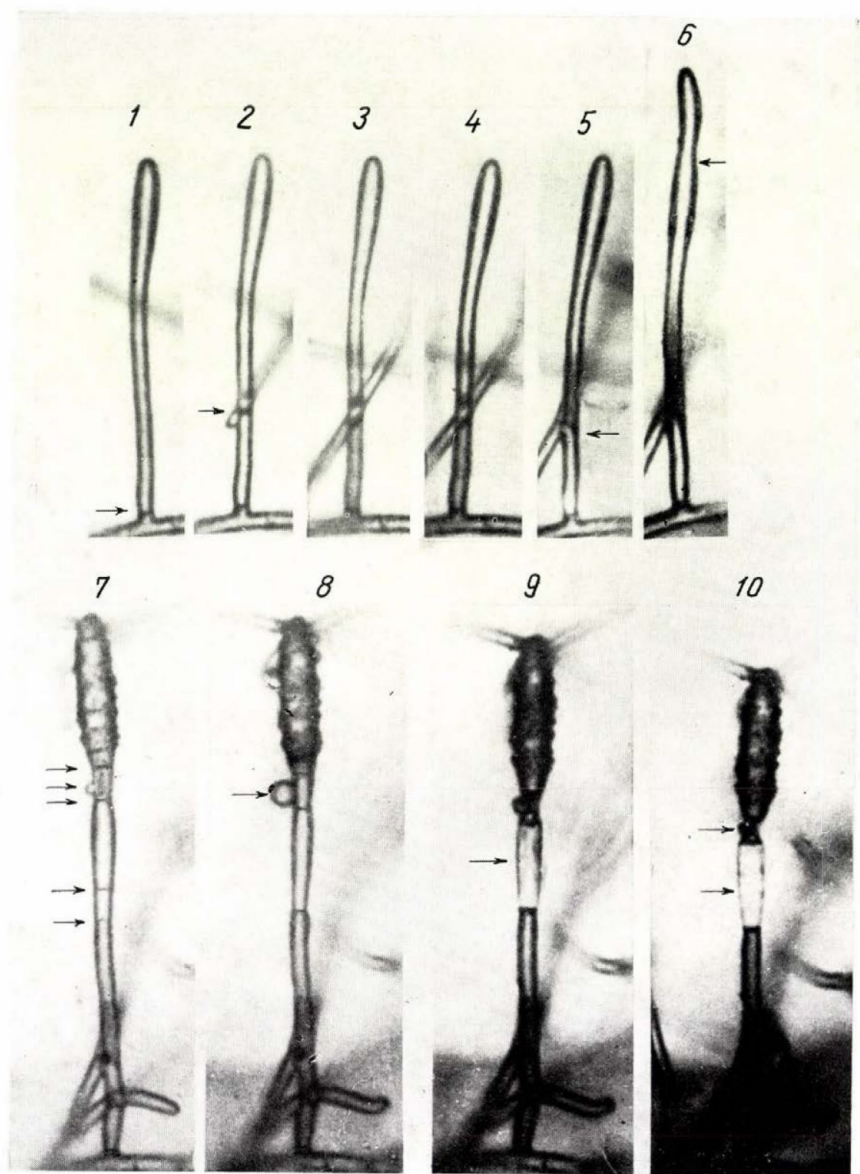


Plate II. Development of a secondary macroconidium of *M. gypseum*

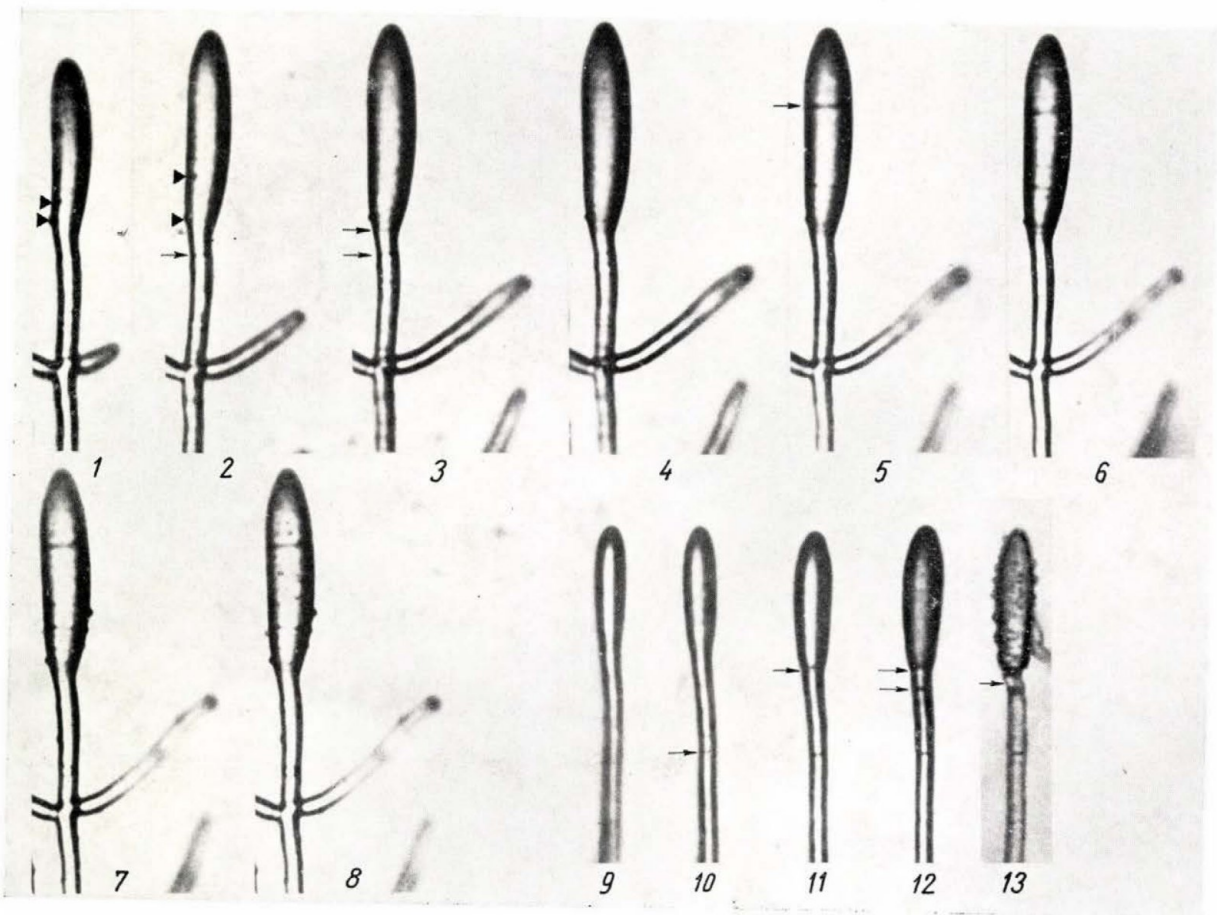


Plate III. Development of macroconidia of *M. fulvum*

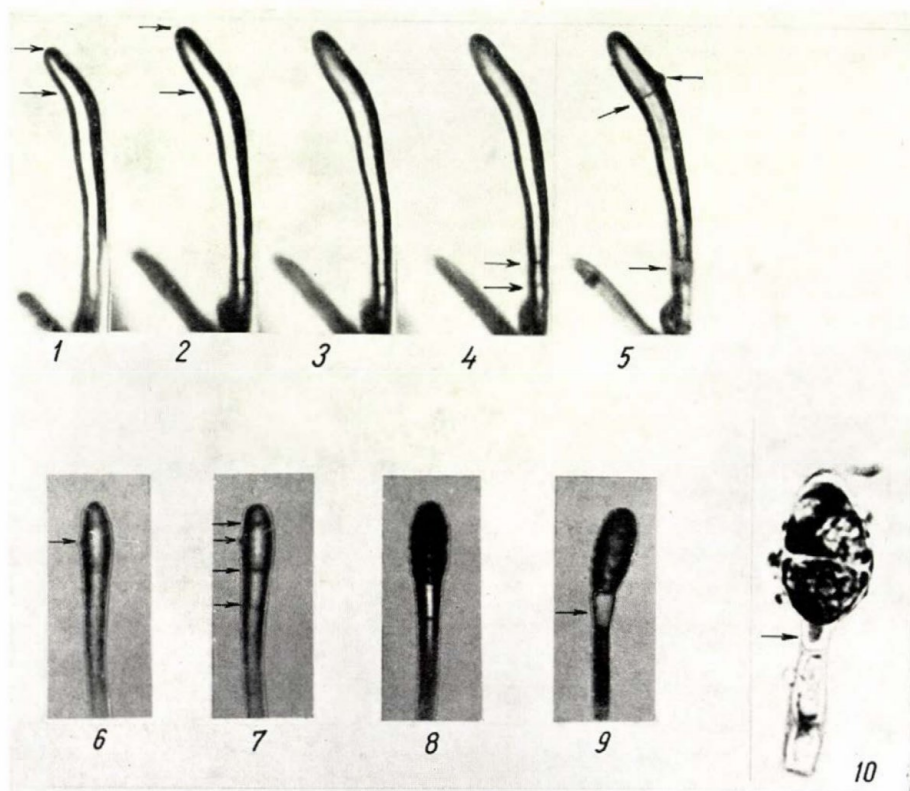


Plate IV. Development of macroconidia of *M. audouinii* (upper row) and *M. nanum* (lower row)

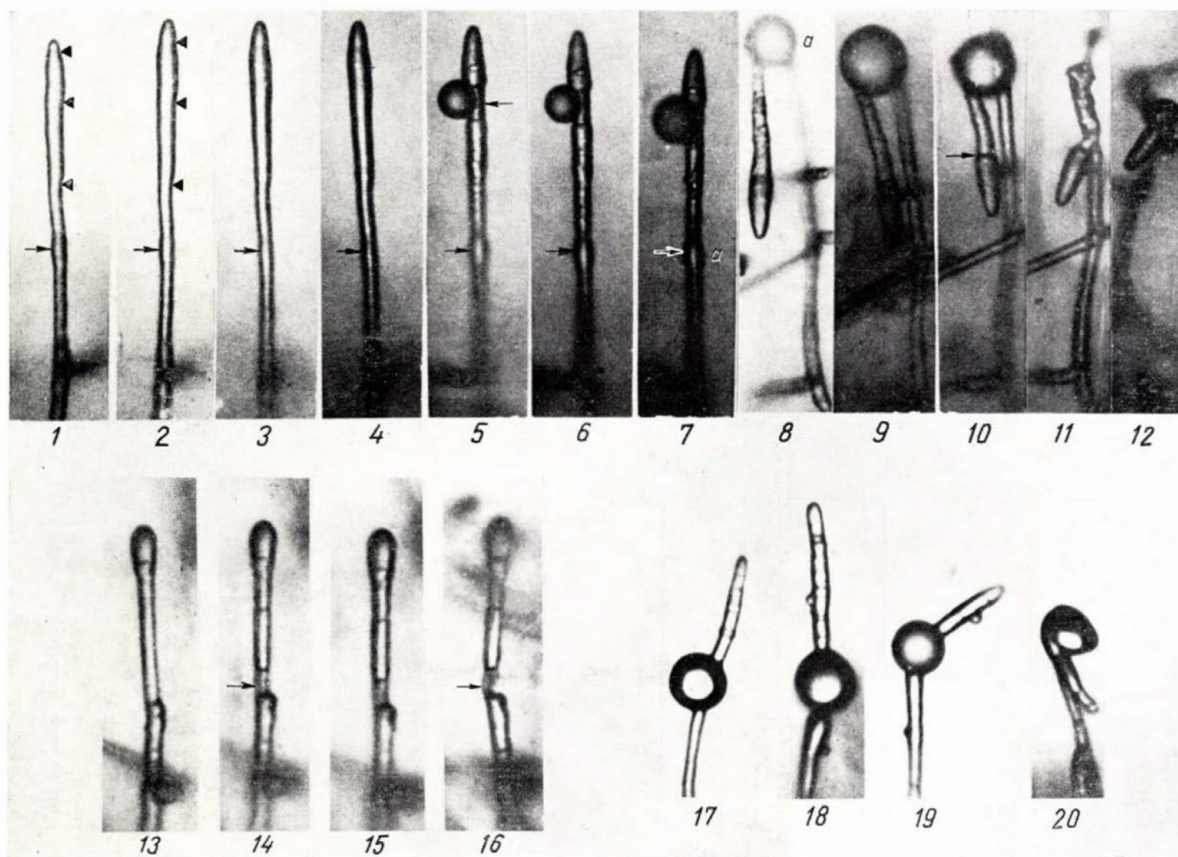


Plate V. Development of the macroconidium of *T. rubrum* (upper row). Separation of the macroconidium of *T. mentagrophytes* (pictures 13-16). Unusual separation of different macroconidia of *T. rubrum* (pictures 17-20)

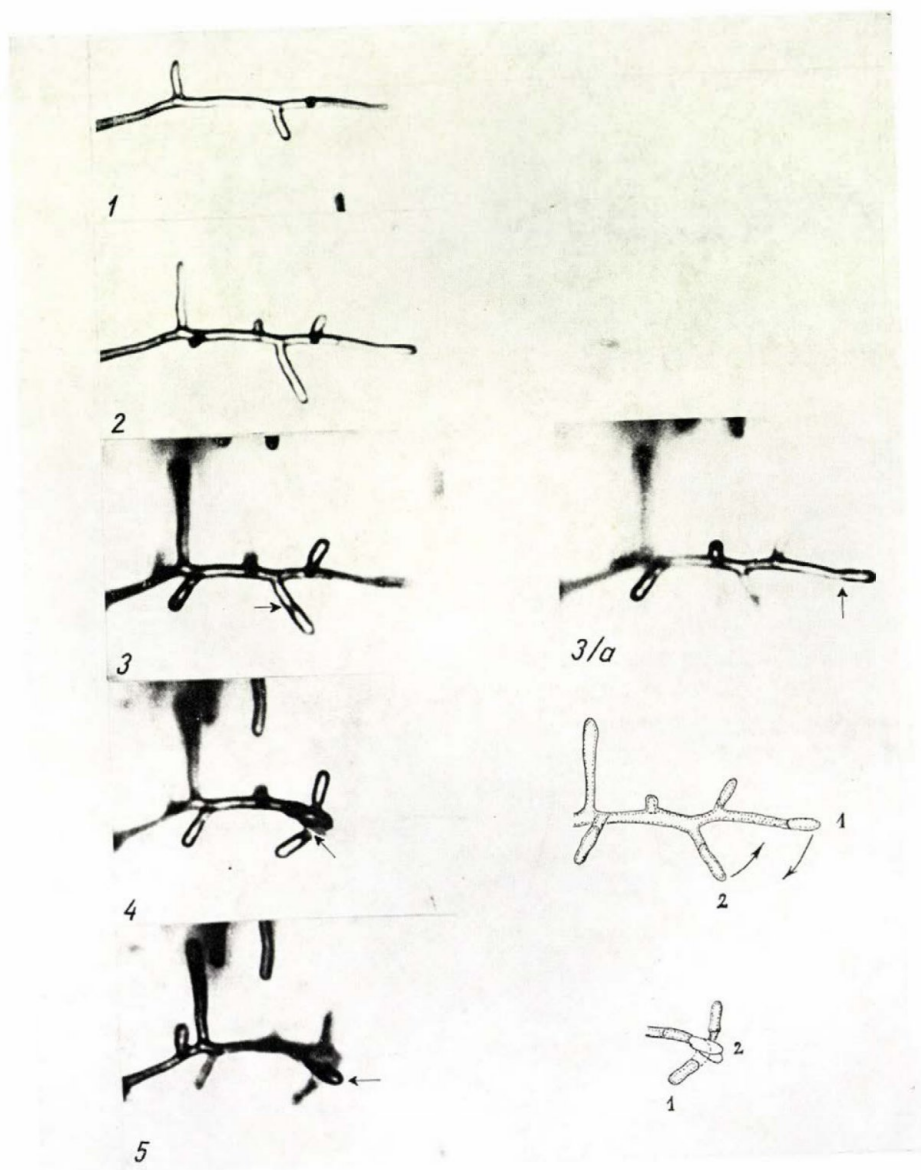


Plate VI. Ontogeny of conidia of *T. terrestre*

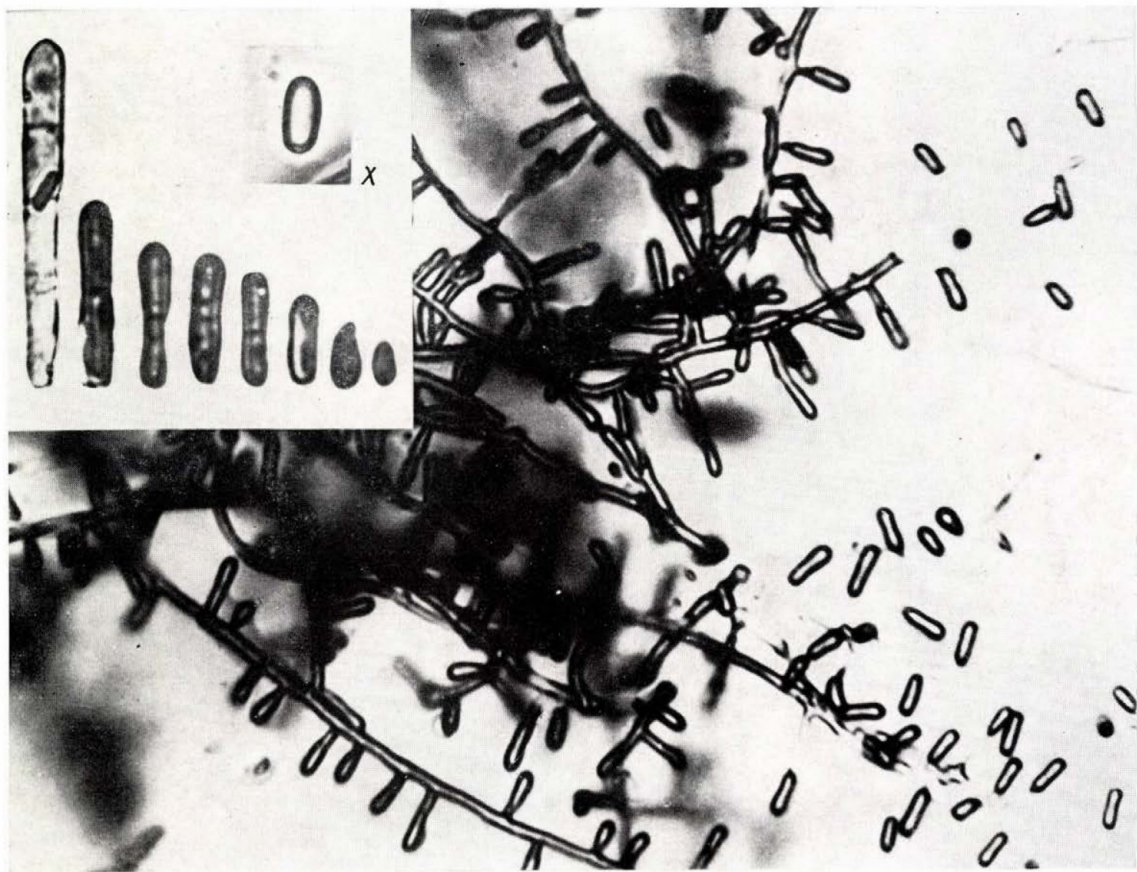


Plate VII. Conidia of *T. terrestre*

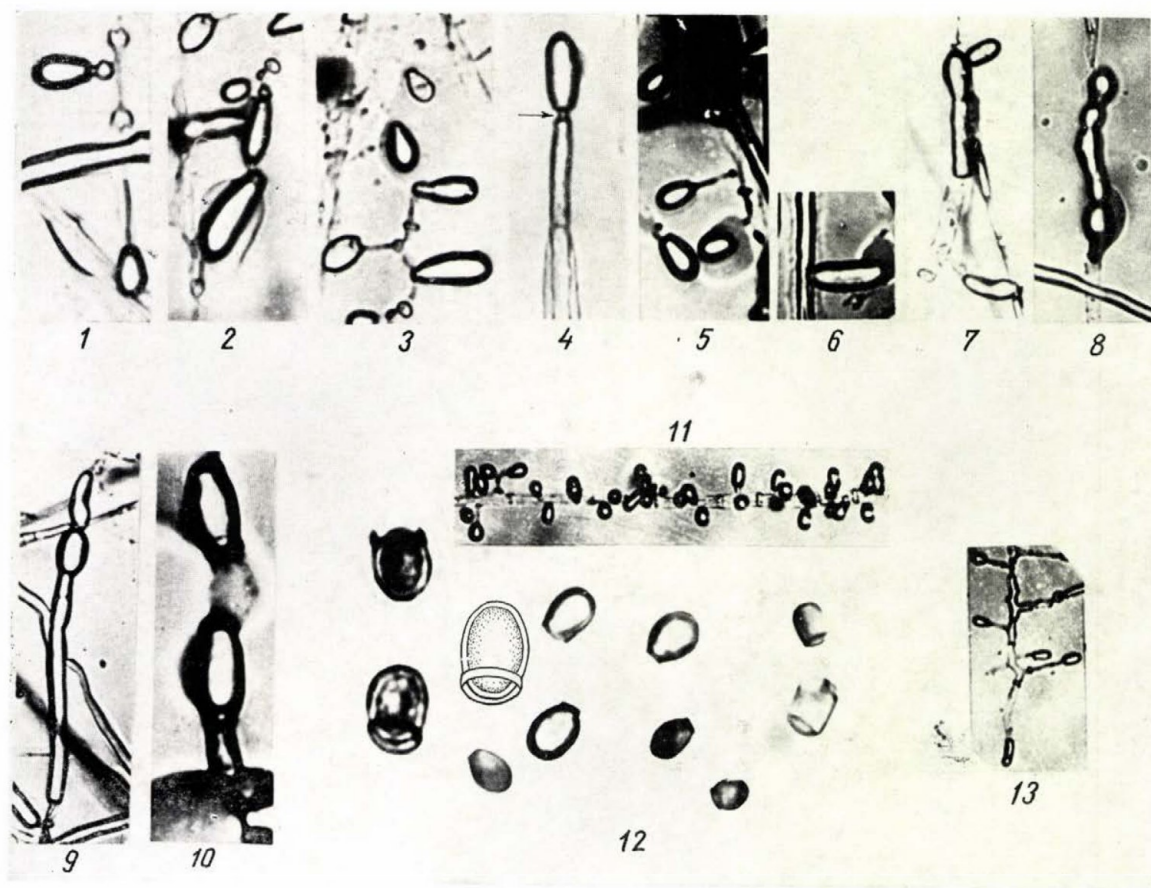


Plate VIII. Microconidia and "gemmae" of genera *Microsporum* and *Trichophyton*

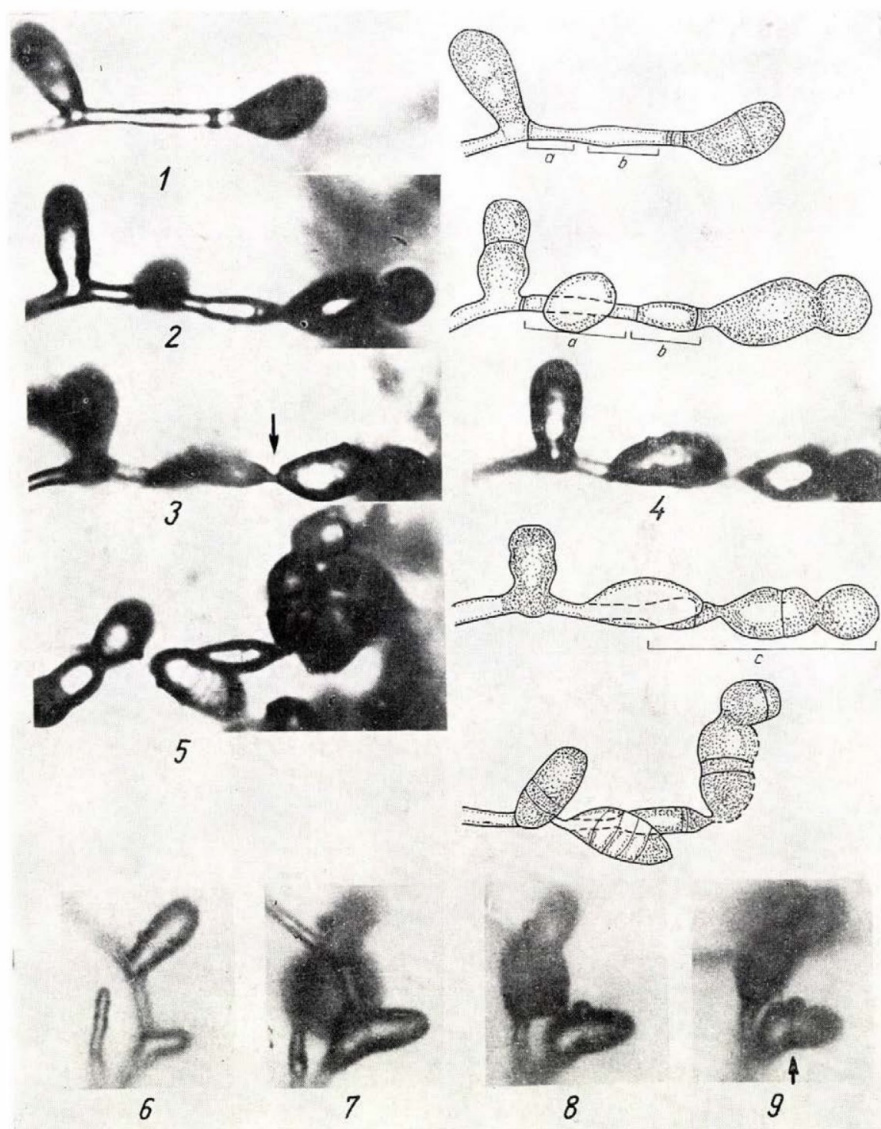


Plate IX. Ontogeny of macroconidia of *E. floccosum*

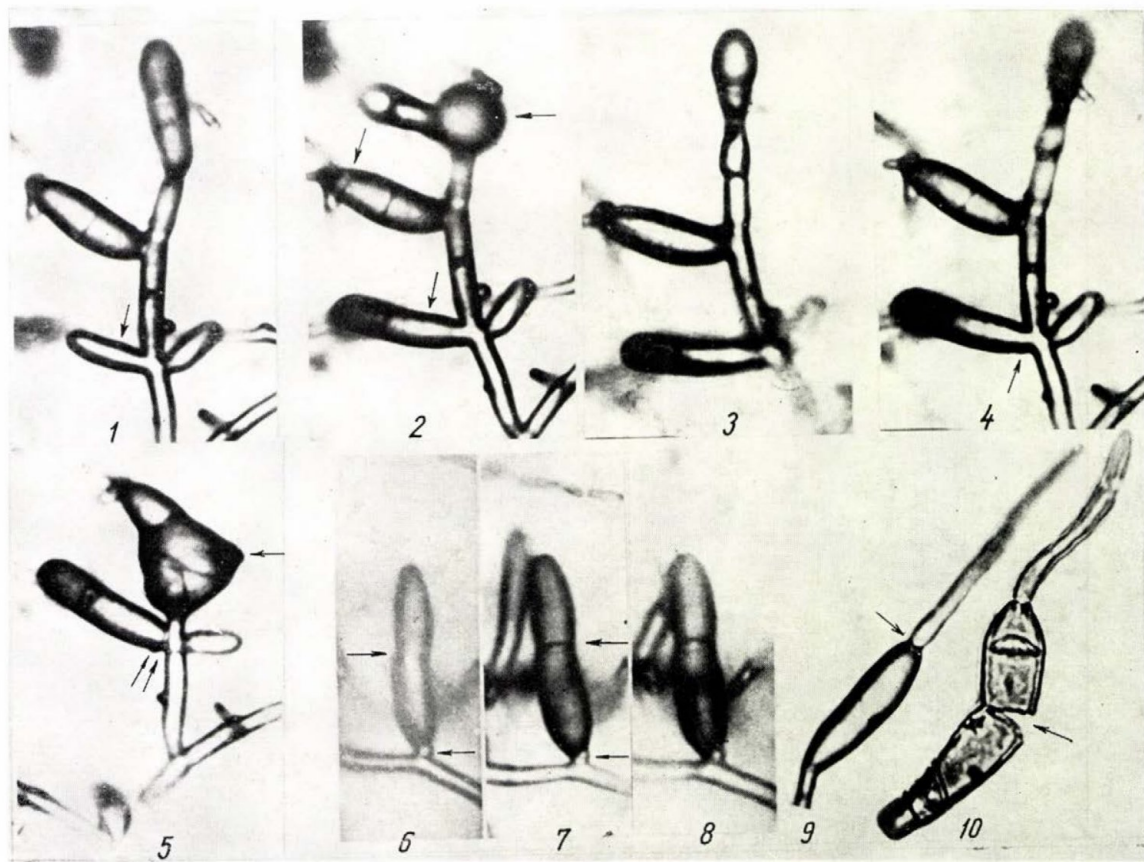


Plate X. Development of macroconidia of *E. floccosum*

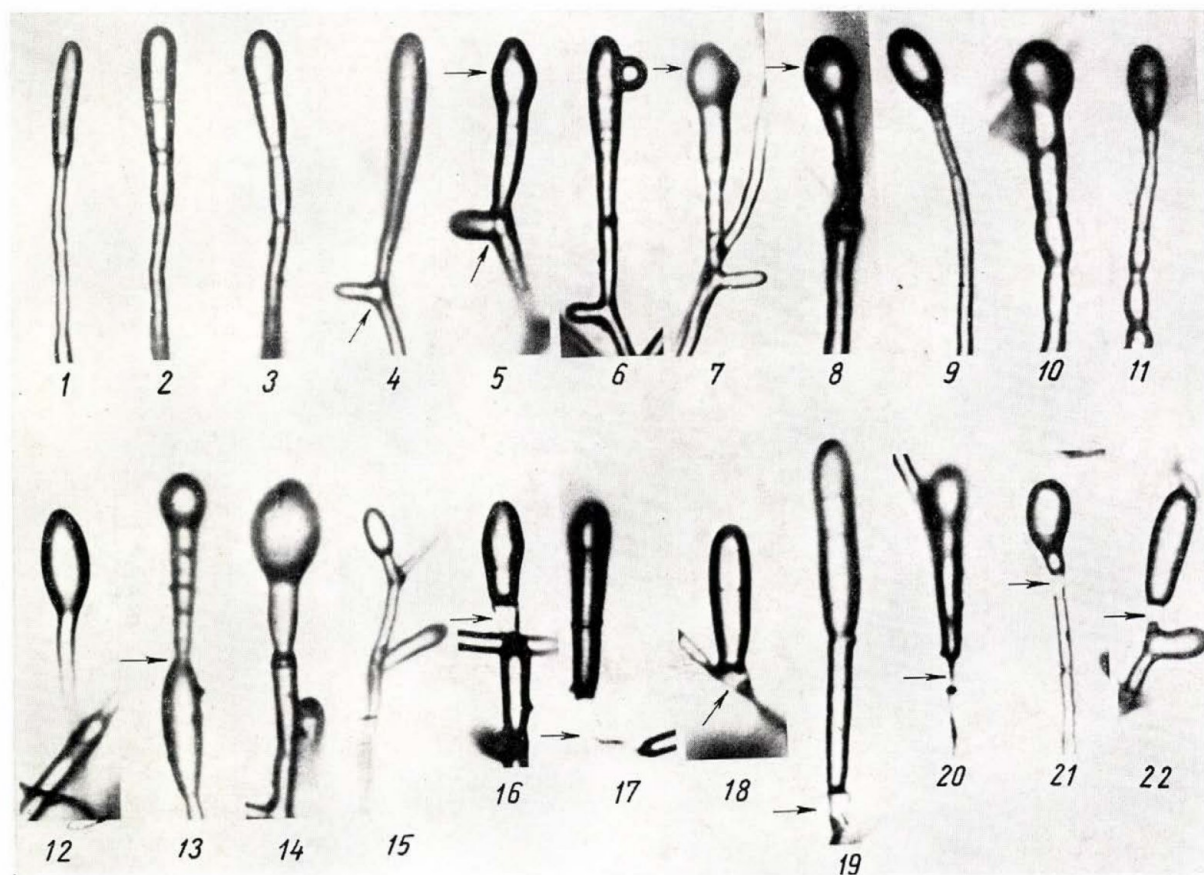


Plate XI. Different types of macroconidia of *E. floccosum*

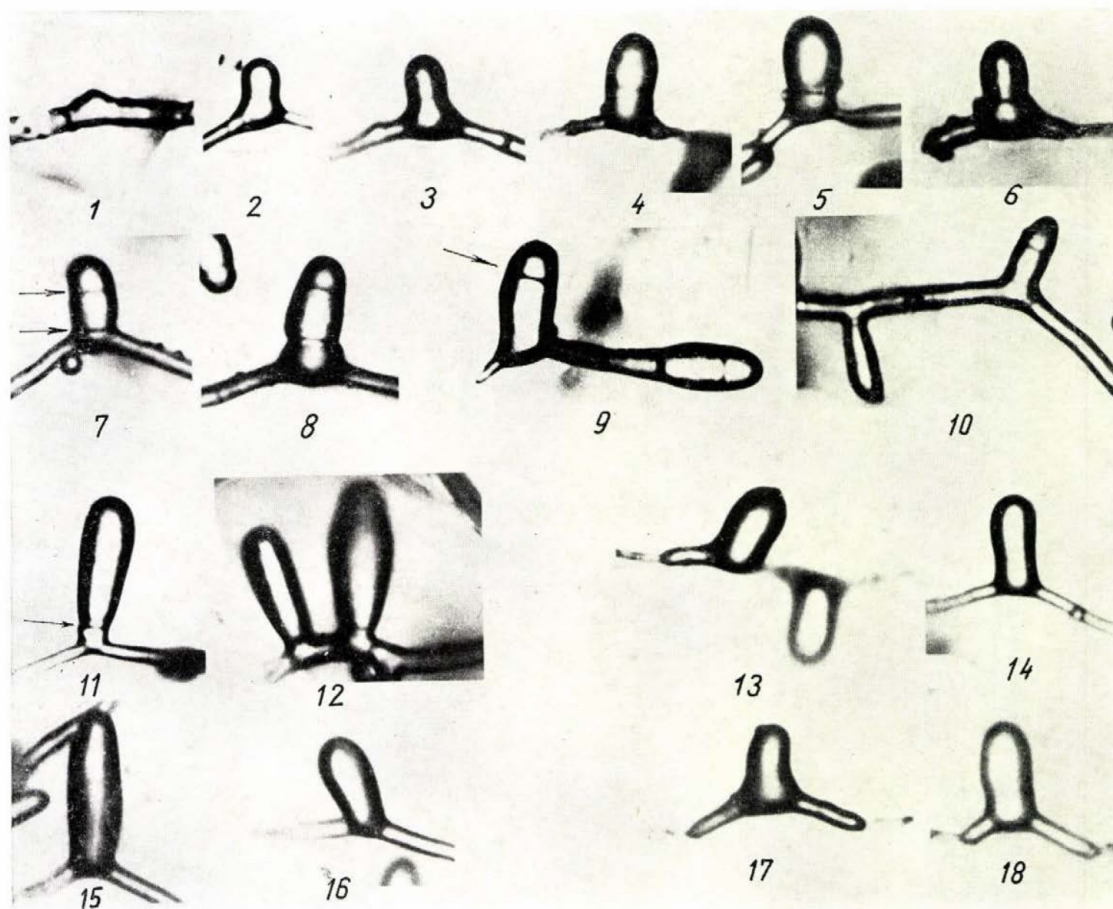


Plate XII. Development of the septa of macroconidia and characteristic detachment of the lateral macroconidia of *E. floccosum*

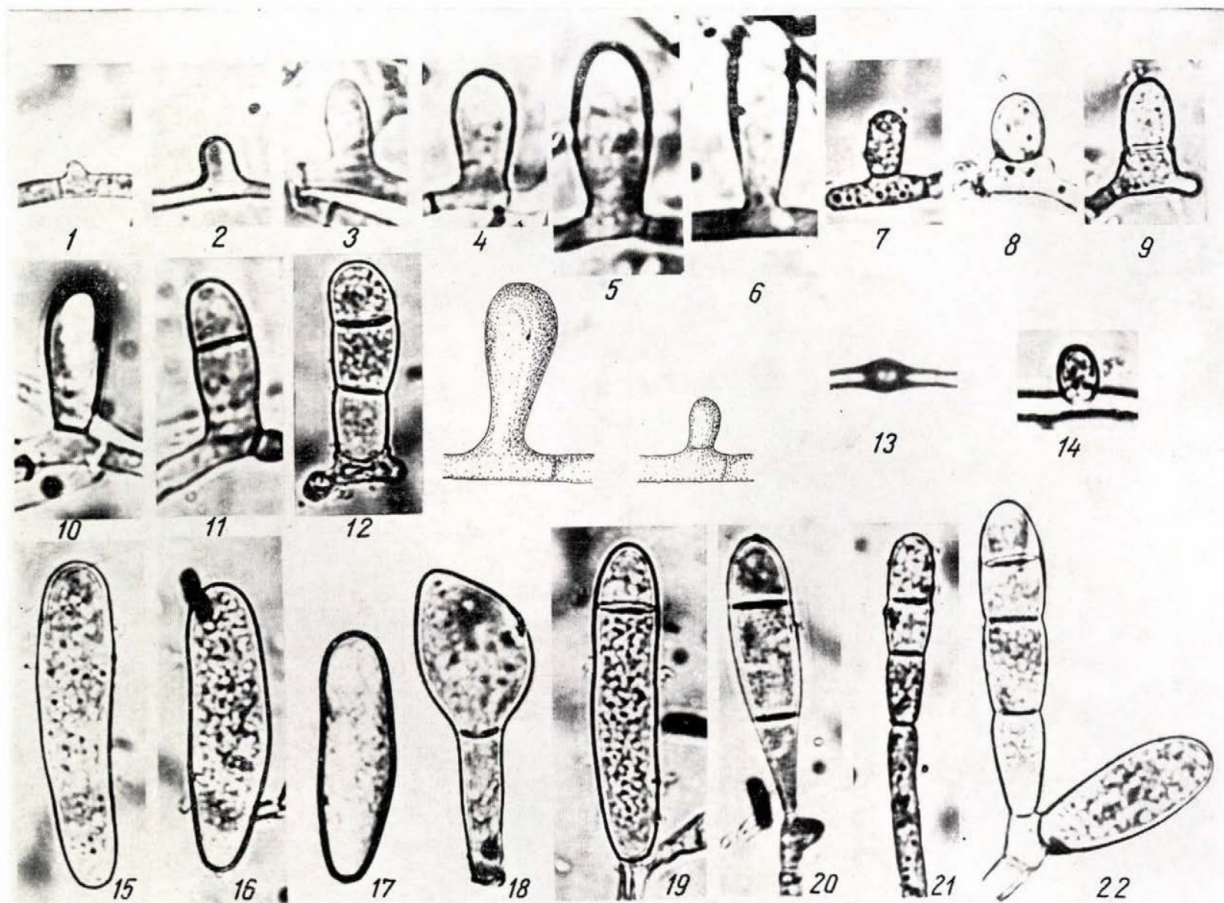


Plate XIII. Inner structure of macroconidia of *E. floccosum* in aqueous preparations

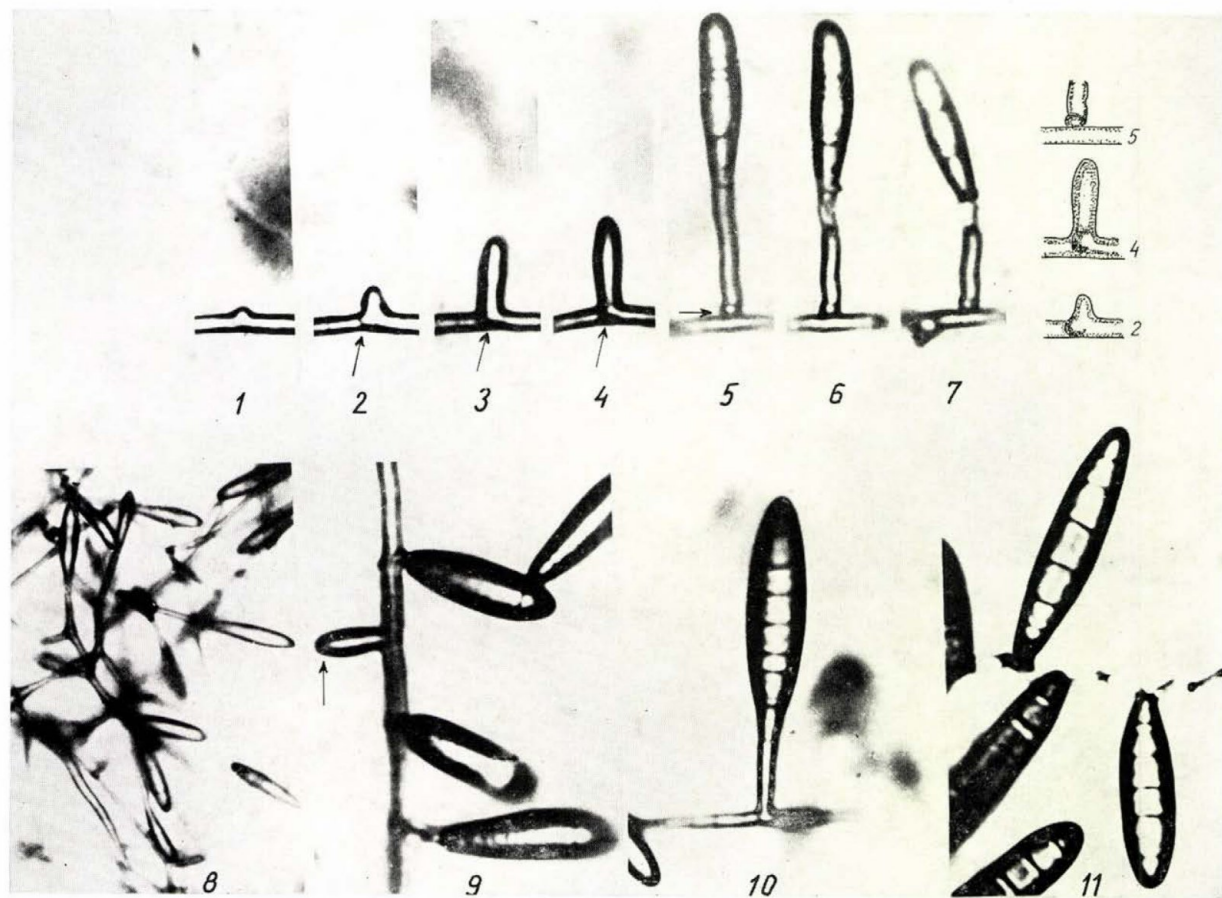


Plate XIV. Ontogeny of macroconidia of *E. ajelloi*

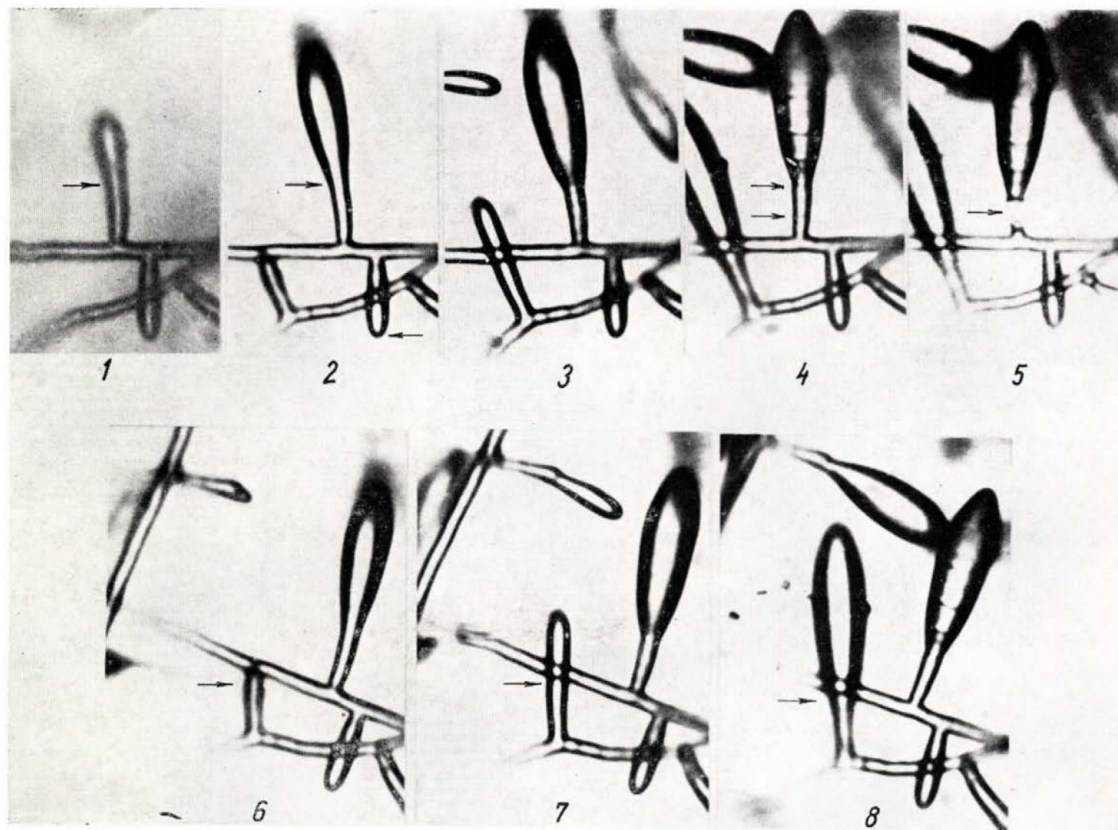
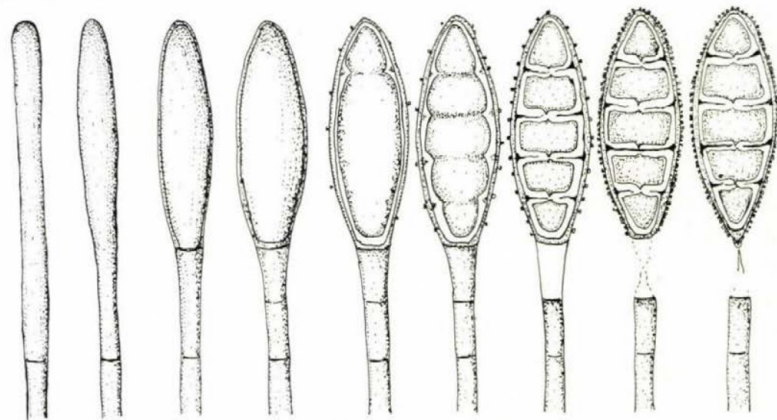
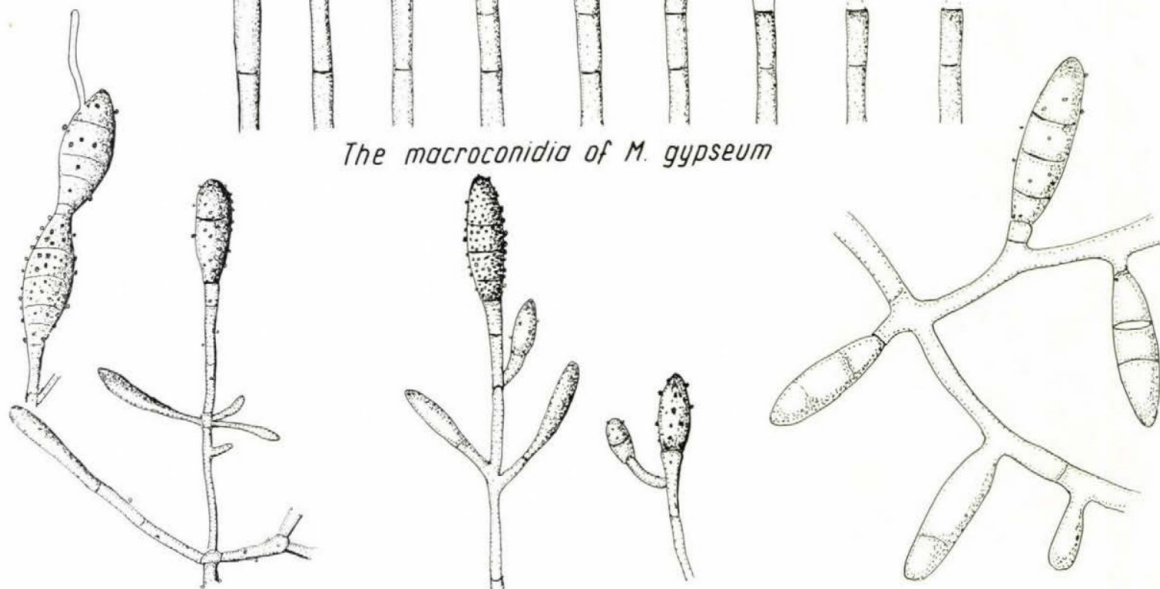


Plate XIV.² Development of the lateral macroconidia of *E. ajelloi*

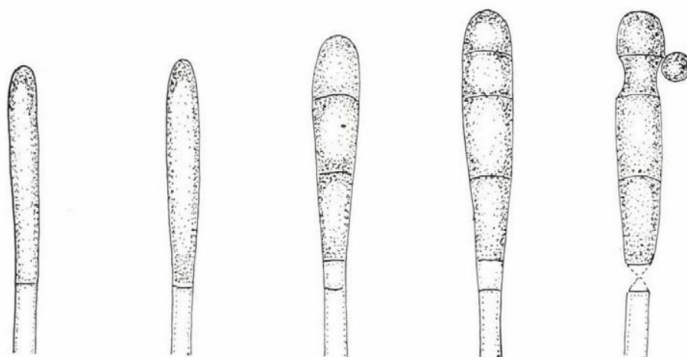
The development of the macroconidium of M. gypseum



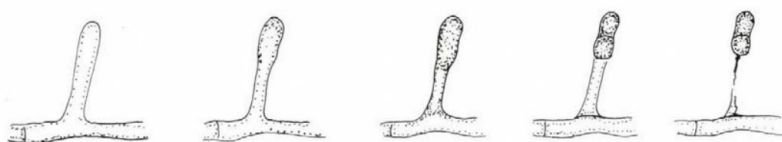
The macroconidia of M. gypseum



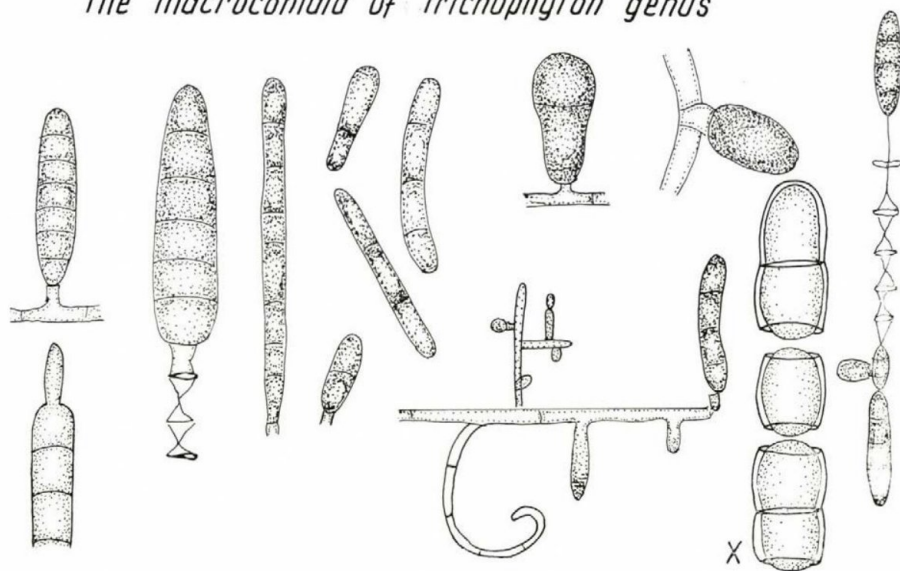
The development of the macroconidium of T. mentagrophytes



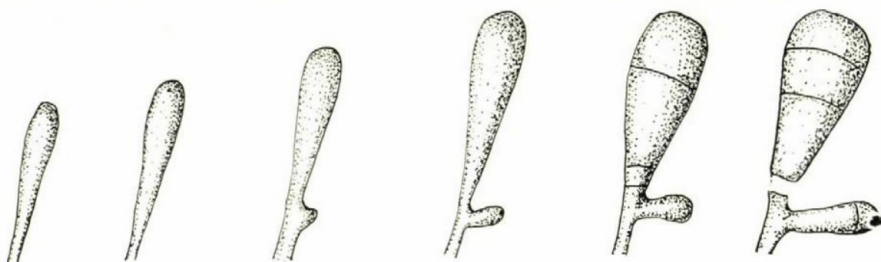
The development of the conidium of T. terrestre



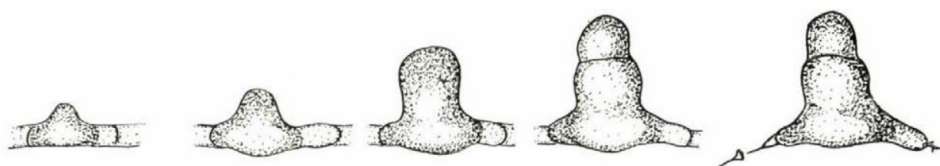
The macroconidia of Trichophyton genus



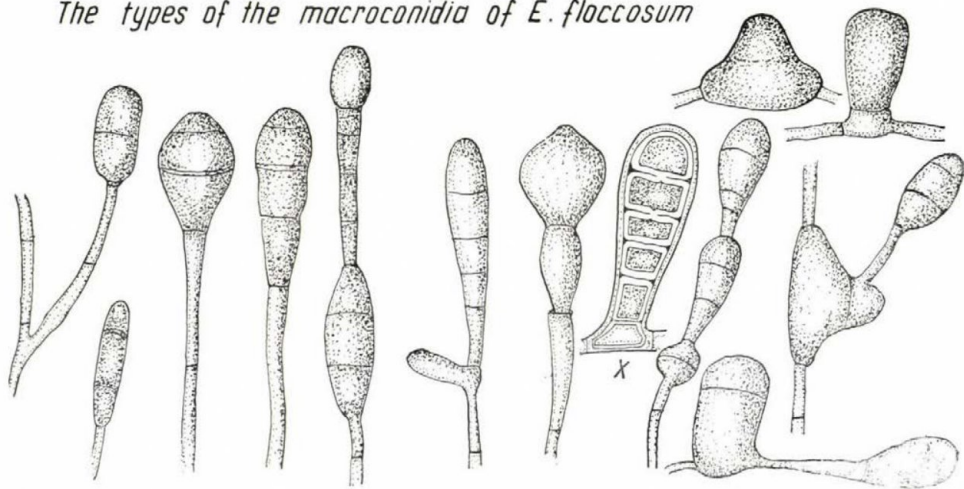
The development of the terminal macroconidium of E. floccosum



The development of the lateral macroconidium of E. floccosum



The types of the macroconidia of E. floccosum



Discussion

The results and recommendations of the Symposium on the Taxonomy of Fungi Imperfecti [11] have been taken into consideration when applying the following terminology. Three terms, *viz.* (i) detaching-cell, (ii) primary septum and (iii) polymeristematic growth are described in detail. The first accurate description of the detaching-cell is given in this paper, and the two others are new terms in literature.

(i) *Detaching-cell*. On the base of, or near to, the conidium (first of all, mc), a thin-walled cell of special function is situated, which is quickly fading and easily torn. Soon the cell wall is absorbed, and the protoplasm of the cell is emptied. Thus, the cell separates the mature mc from the intact supporting structure. The direction of protoplasm migration or the way of its absorption may be variable: (a) it may empty through a newly-formed pore of the cell wall; (b) it may empty through the pore of the upper (mc-sided) or the lower (conidiophore-sided) septum, probably by a hypothetical suction effect.

(ii) *Primary septum* is a septum developing usually but not exclusively, at the boundary of the middle and upper third of the mc, or in the lower part of the apical area of the mc, preceding the other septa. It appears in the early stage of mc development.

(iii) *Polymeristematic growth*. Numerous meristems are situated on the base, apex and on many sites of the corpus (*i.e.* basally, apically and intercalarily) of conidiophora and of mc, which may come into action simultaneously or successively.

VUILLEMIN and BRUMPT — and other medical mycologists — have termed the conidia of dermatophytes aleuriospores. In connection with these organs insufficiently supported ideas have been published resulting in a total confusion. Some authors believed that these conidia would appear by the concentration of protoplasm, and so they could not be real conidia. Furthermore, it was believed that they would have no detaching mechanism so they would stay in close connection with their supporting structure until its complete destruction. Others have distinguished between the aleuriospores, and lateral spores, suggesting that aleuriospores are released only after the destruction of holding hyphae, whereas lateral spores can be released without destroying the hyphae.

Considering the erroneous interpretations, furthermore, as the term "aleuriospore" refers to the way of separation but not of the development of conidia, it can be disregarded when discussing the conidium-ontogeny of dermatophytes. All the more so, as it has been shown in the present work that the conidia of *Dermatophyton* fungi have a well-definable detaching mechanism and neither the whole thallus nor a part of it have to be destroyed to allow a separation of conidia.

The most important is to distinguish between "blastic" and "thallic" developmental main-types in connection with conidium ontogeny.

As regards the development of macroconidia of the genus *Microsporium* the following can be stated.

(i) Development of the macroconidia of some species (e.g. *M. gypseum*) is rapid. The minimum time necessary for complete development under optimum circumstances is 24 hr. Other species (e.g. *M. audouinii*) grow slower.

(ii) Macroconidia arise on the apex or on the side of simple or branched conidiophores. Meristems are located in these areas.

(iii) Meristems situated on the apex and also on different points of the corpus of macroconidia secure an even, simultaneously longitudinal and transversal growth.

(iv) All the species have complete detaching mechanisms.

(v) The phases of development of the mc in chronological order are as follows.

A. On the meristem, i.e., on a point of one cell of a hypha a unidirectional bulge appears, which may develop further in different ways. (a) A longer or shorter lateral conidiophore develops and, subsequently, its apical region begins to swell and takes the shape of a young mc. On the apex of unbranched conidiophores, similar phenomena can be observed. (b) A short cervical part develops. This may be considered a short conidiophore branching from the main branch or, more likely, be regarded as the detaching-cell of a lateral mc which has no conidiophore. This short neck will later serve as a detaching-cell. The thin, hypha-like mc-initial appears to swell evenly in its full length after having reached full length. (c) The lateral mc appears immediately on the conidiophore. In this case, it starts as a swollen tube from the first minute and the wide basal cell of the mc is situated on the side of the hypha.

B. The detaching-cell appears.

C. The primary septum appears.

D. The other septa appear, the wall of the corpus becomes thick, with warts on it.

E. The outer surface of the wall becomes considerably warty.

F. The detaching mechanism performs the separation of conidia.

As to the development of the macroconidia of the genus *Trichophyton*, features partly similar partly dissimilar to those of the genus *Microsporium* may be met with. The macroconidia of the former are definitely less differentiated, and less separated from the supporting structure. Their structures (the thickness of the wall of the mc and the characteristics of its surface, etc.) are much simpler, furthermore, the undifferentiated, occasional character of the shape and size of the complete mc markedly differentiate them from members of *Microsporium*. In our drawings, the genus *Microsporium* has been represented by *M. gypseum* only because this species produces macroconidia

characteristic of the genus and is stable under all circumstances. The smooth-walled macroconidia, characteristic of *Trichophyton*, vary in size and often in shape, to such a degree that, when identifying the species of this genus, one has to examine carefully some other peculiarities as well. For this reason, we present all the main forms of mc characteristic of the genus *Trichophyton* (Plate XVII).

Trichophyton also produces terminal and lateral macroconidia. The latter start generally as wide-based widenings, which develop into thick, sausage-like tubes in several hours. No septa can be observed in these tubes. Septation, the direction of which is accidental, and development of the detaching-cell start at about the same time.

Unlike with *Microsporum*, in the genus *Trichophyton* it cannot be predicted whether or not on a hypha-tip will develop an mc. Practically, any hypha-tip may transform into a terminal mc. Furthermore, the culture has to reach a certain age when as a sign of the next stage of maturation, the macroconidia appear and take their final shape. It seems reasonable to suggest that in *Trichophyton*, part of the culture must be destroyed for the development and detachment of macroconidia. Obviously, some macroconidia do not obtain their final, cylindrical, swollen form until part of the supporting structure has been absorbed. In contrast to the genus *Microsporum*, where usually one single special cell accounts for the separation, in *Trichophyton* often the autolysis of a whole cell-row is needed for this. Furthermore, part of the branching conidiophore (fertile hypha) is soon absorbed, even in the case when detachment is started by the absorption of the detaching-cell. In addition, the great variability of "gemmae", i.e. protoplasmic concentrations, and the constant changes in this picture explain why so many different theories have been proposed. The *Trichophyton* species do not seem to "remember" their asexual organs, their differentiation, the role of the detaching-cell, etc., in contrast to the behaviour of *Microsporum* species and especially to that of *M. gypseum*, where almost all the components of the thallus are well-delimited and permanent as to shape and function. The incomplete detachment and the very simple and irregular shape of the macroconidia might perhaps be explained by this assumption. One cannot find any order in this irregularity: the length, thickness, bending, swelling etc., seem to be the result of an adaptation to a momentary situation, in order to avoid obstacles during development.

The development of the macroconidia of *T. rubrum* also demonstrates the incompleteness of conidium detachment (Plate XV).

In spite of these facts one can observe certain regularities, viz. (i) in certain species (e.g. *T. terrestre*) macroconidia develop much faster (roughly in 30 hr) than in other (e.g. *T. verrucosum*); (ii) the conidia arise on the tip of simple or branching conidiophores, or laterally at sites where meristems are present; (iii) meristems are situated also on the apex of mc, furthermore,

in different areas of its corpus, ensuring an even and simultaneous longitudinal and transversal growth; (iv) the short and swollen lateral macroconidia are differentiated organs, often having regular detaching-cells. The septa take shape after the tube (the young, empty corpus of the mc) has developed.

Traces of a former fixation are left behind on the bases of the conidia of *Microsporum* as well as *Trichophyton* species. The earlier the connection between the conidium and the supporting structure breaks off the better it is seen. Very young conidia — broken off artificially — hold a regular collar on their base, turning it away from the detaching-cell or the conidiophore. The behaviour of *T. terrestre*, a species having neither distinct microconidia nor macroconidia, somewhat deviates from that of other *Trichophyton* species. Furthermore, its conidia include some transitional forms. It produces "gemmae" less frequently than the other species do. On the other hand, it produces numerous conidiophores, which consistently rise to conidia *viz.*, each branching is the equivalent of a conidiophore, on the apex of which a conidium will soon develop. This development involves the growth to a given length of the conidiophore and its moderate apical swelling. Subsequently, both the septum separating the conidiophore (strictly speaking, one of the lateral branches of the conidiophore) from the basal hypha and that separating the conidium from the conidiophore, appear almost simultaneously. The area between these two septa is completely absorbed, and a large part of the supporting-structure autolyzes. The old denomination "fungus having aleuriospores" applies best to this species. The conidia which fall down do not seem to tear away any residue of the cell-wall from the supporting structure.

The *Microsporum* and *Trichophyton* genera form also microconidia, the development of which is essentially similar to that of the macroconidia, though at a far simpler level.

In the genus *Epidermophyton* two species: *E. ajelloi* [1, 3] and *E. floccosum* were examined.

E. ajelloi (earlier name, *Keratinomyces ajelloi*), owing to its diffuse behaviour, has been ranged among all the genera of dermatophytes. It was classified as *Trichophyton* on account of its perfect form (*Arthroderma*); as *Epidermophyton* in view of the total and regular absence of microconidia; and finally, as *Microsporum* due to the morphology of its macroconidia. Obviously, the overall development of its macroconidia corresponds to that characteristic of the genus *Microsporum*.

E. floccosum is a species which shows an unusual variability in producing macroconidia.

We have failed to confirm the results of CAPRILLI *et al.* [12], who suggested that the macroconidia of *E. floccosum* show a special development, as the apical cell is always the oldest, and the basal cell the youngest. This would be a mechanism essentially different from that seen in the genus *Micro-*

sporum. In fact, development of the macroconidia of *E. floccosum* is similar since (i) it proceeds rapidly; (ii) macroconidia rarely arise on the apex of the conidiophore, more frequently they grow laterally; (iii) meristems are situated on the apex of the mc and also at different sites of its corpus, ensuring an even and simultaneous longitudinal and transversal growth; (iv) the phases of development of the mc in chronological order are as follows.

A. On the conidiogenous locus, which may be a point, an area or a zone of a hyphal cell, an irreversible outgrowth appears. From this (a) a longer or shorter conidiophore develops, from the whole or a part of which an mc originates. The large swelling of the apical cell appearing later is highly characteristic; (b) the lateral mc originates directly, showing an even longitudinal and transversal growth.

B. Septa and detaching-cell(s) are formed.

C. The detaching mechanism comes into action. The detaching-cell of the terminal macroconidia is similar to the former ones. Detachment of the lateral macroconidia is characteristically bilateral (Plates XII and XVIII).

D. Autolysis of macroconidia begins within 1–2 days. This phenomenon is typical of this species only.

Plate IX. shows a definite longitudinal development of the conidiophore in some parts, and its transversal development in other parts, while a growth starts from the apex of the terminal mc as well. A lateral mc develops on the extended area of the conidiophore, and besides, an intercalary mc originates simultaneously as a result of transversal growth. Thus, meristems are situated at different sites of the conidiophore as well as on the base and apex of the mc, which come into action simultaneously or successively. The longitudinal growth of the conidiophore to such an extent — after the full development of the terminal mc — has not been observed in other species. Some “basauxic” growth can be seen on the base of the conidiophore of *E. ajelloi* as well (Plate XIV).

In conclusion, (i) the genera *Microsporum*, *Trichophyton* and *Epidermophyton* have a holoblastic conidium ontogeny; (ii) the investigated species exhibit polymeristematic growth; (iii) delivery of conidia occurs by means of a special detaching mechanism, by an autolysis of the detaching-cell(s); (iv) the macroconidia have a primary septum; (v) the species under study also form chlamydospores including “gemmae” and “persistent-organs” strikingly similar to the macro and microconidia as investigated in aqueous preparations.

Dermatophytes may be ranged into the section III of the system of HUGHES [5] and into group III/C of TUBAKI's [13] system.

The development as well as some characteristics of the macroconidia are illustrated by drawings in Plates XVI–XVIII, where those marked by x show the inner structure of the macroconidia.

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THE ROLE OF INTERFERON IN INTERFERENCE AND AUTO-INTERFERENCE ELICITED BY NEWCASTLE DISEASE VIRUS

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Summary. Newcastle disease virus (NDV) strains interfere in different degree with the growth of the velogenic NDV strain Texas GB (homologous interference) and Sindbis virus (heterologous interference) in chick embryo fibroblast cells. Homologous interference was elicited by interferon-producing live or UV-inactivated strains and non-interferon-producing live or beta-propiolactone-inactivated strains and it was not influenced by actinomycin D. Thus, interferon had apparently no role in homologous interference of NDV. The growth of Sindbis virus was, however, much more inhibited by interferon-producing live or UV-inactivated NDV strains than with non-inducing ones and the interference was reversible by actinomycin D. Thus heterologous interference is apparently mediated by interferon. In chicken cells infected with the mesogenic NDV strain H, virus yields were 50 to 100 times lower at multiplicities of infection above 0.1 p.f.u./cell than below it. The interferon formed during infection played no role in auto-interference, but may well be held responsible for the mild cytopathic effect observed.

Interference between certain strains of Newcastle disease virus (NDV) [1–4], NDV and arbovirus [5] and certain group A arboviruses [6] has long been observed. The conditions and mechanism of interference between different NDV strains were studied in great detail by BRATT and RUBIN [4]. ORLOVA *et al.* [7] explained the phenomenon by a suppression of the RNA synthesis of the superinfecting NDV. All authors quoted above excluded the role of interferon (IF) in interference phenomena, although ZEBOVICZ and BROWN [6] used an IF-sensitive virus (eastern equine encephalitis) in their system.

Concerning the mechanism of auto-interference, opinions are conflicting. HERMODSSON [8] attributed the auto-interference of the scarcely IF-sensitive NDV in calf kidney cells to IF, whereas SREEVALSAN and LOCKART [9] excluded the mediating role of IF in auto-interference of the highly IF-sensitive western equine encephalitis (WEE) virus.

The present studies were carried out to clarify the role of IF in homologous interference of NDV strains, heterologous interference of NDV and Sindbis virus and auto-interference of the NDV strain H.

Materials and methods

Materials. Actinomycin D was produced by Reanal Pharmaceutical Works (Budapest) and beta-propiolactone (BPL) from the Fluka A.G. (Buchs, Switzerland).

Virus strains. Source, propagation and titration of the NDV strains (LaSota, Ulster, L, H, Texas GB) and Sindbis virus were the same as described previously [10].

Virus inactivation. Inactivation with UV-light was carried out according to GANDHI and BURKE [11], and with BPL according to NEFF and ENDERS [12].

Cell culture. Primary chick embryo fibroblast (CEF) cultures were prepared from 9-day-old embryos as described previously [13].

Production of IF for cell pre-treatment and titration of IF. Both procedures were carried out as described earlier [10]. IF values were expressed in international units.

Table I

Growth of NDV-Texas and Sindbis viruses in IF-producing and non-producing systems

Pretreatment of cells****		Virus yields (in per cent) and time of superinfection*							
		NDV-Texas**				Sindbis***			
		+4.5	+2.4	+0.5	-1	+4.5	+2.5	+0.5	-1
None	—			100				100	
	Act.	50	60	73		36	48	100	
IF	—	55				0.05		5	
	Act.	45				93			
Ulster	—		0.14				1.8		
	Act.		0.24				45		
L-UV	—	0.64	0.54	3.5	68	0.03	0.25	5	12
	Act.		0.92			70	76		
LaSota	—	0.01	0.02	0.1	0.6		42		
	Act.		0.03				84		
L-BPL	—	1.4	0.72	6.8	44	34	38	44	48
	Act.		0.34			94	72		

* Cells were superinfected at 4.5, 2.5 and 0.5 hr after and 1 hr (—1) before the interfering treatment

** Cells were superinfected with 1 p.f.u./cell of NDV-Texas and virus was harvested at 15 hr after infection; 100% = 2.2×10^7 p.f.u./ml

*** Cells were superinfected with 4 p.f.u./cell of Sindbis virus and virus was harvested at 13 hr after infection; 100% = 2×10^9 p.f.u./ml

**** Cells were pretreated with 80 IU/ml of IF or with NDV strains at a m.o.i. of about 100 p.f.u./cell; 0.12 µg/ml actinomycin D (Act) was used

Results

Demonstration of homologous interference. Growth of the velogenic (virulent) Texas strain of NDV was inhibited by the UV-inactivated mesogenic strain L (L—UV) and by strain H (Fig. 1). To prevent interference in acting at the level of virus adsorption [3, 14], treatment of the cells with the interference-inducing L-UV or H strain was carried out after infection with the Texas strain. No notable inhibition of the growth of strain Texas was found if its multiplicity of infection (m.o.i.) was too high. At less than 5 p.f.u./cell

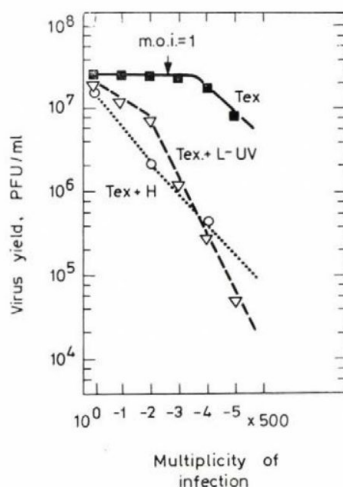


Fig. 1. Effect of subsequent infection with strains H and L-UV on the yields of NDV-Texas strain in CEF. Yields in CEF infected with different m.o.i. of strain Texas ■—■; yields of Texas strain in cells subsequently infected with strain H ○·····○, or L-UV ▽-----▽ 30 min after Texas strain

the yield of Texas strain was however reduced by more than 90% in cells previously infected with either L-UV or strain H. As both L-UV and H are IF-inducers [10], it seemed important to clarify the role of IF in the inhibition of virus growth.

Role of IF in homologous and heterologous interference. CEF cultures were infected with IF-inducers such as live, avirulent strain Ulster and L-UV, and with non-inducers like strain LaSota and BPL-inactivated L-strain (L-BPL). L-UV is a good inducer, whereas L-BPL does not induce IF (unpublished data). The cultures previously infected with the interfering viruses were superinfected with strain Texas of NDV or Sindbis virus at different times. The Texas strain is not sensitive to IF, whereas the Sindbis virus is highly sensitive, about 5000 times more so than NDV [10]. Part of the infected

cultures was treated with actinomycin D, to inhibit an eventual IF synthesis. Results are summarized in Table I.

Treatment of CEF cultures with 80 IU of IF 4.5 hr before infection reduced the yield of NDV 2 times while that of Sindbis virus 1000 times, and this inhibition of virus growth by IF could be reversed with actinomycin D. Thus, in any further instance when inhibition of the growth of the superinfecting virus was reversed by actinomycin D, the mediation of interference by

Table II
*Effect of m.o.i. of the interfering virus
on the degree of interference*

Interfering		Yield of superinfecting virus	
Virus	m.o.i	NDV-Texas, per cent	Sindbis, per cent
None	—	100	100
LaSota	150	0.03	n.d.
	1	20	n.d.
L-UV	230	0.5	0.25
	23	13	10
	2	95	100

Conditions of experiments were as in Table I

n.d. = not done

IF was regarded as proven. The growth of Sindbis virus was markedly inhibited by both the IF-producing live Ulster strain and L-UV, but the non-IF-producing strains did not suppress it. The interference phenomena were almost completely reversed by actinomycin D. Interference by L-UV was the more pronounced the longer time was allowed to elapse between infection with the interfering virus and superinfection, and the level of interference (*i.e.* the degree of inhibition of Sindbis virus growth) corresponded to the degree of inhibition of virus growth measured in the IF-pretreated cells.

In contrast, growth of the NDV Texas strain was markedly inhibited by all the strains tested, whether live or inactivated and whether or not IF was induced. Actinomycin D had no influence on the interference phenomena. Live strains had a more distinct inhibitory effect than inactivated ones; the latter did not inhibit growth of the Texas strain unless they were applied prior to it, whereas the live LaSota strain interfered with growth even on subsequent application. Interference was the more marked the longer time was allowed to

elapse between infection with the interfering and the Texas strain; but it did not further increase after 2.5 hr. Accordingly, while maximum heterologous interference against Sindbis virus required several hours, development of homologous interference against NDV took only 1–2 hr.

Effect of the multiplicity of the interfering virus on the degree of interference. Results are shown in Table II. The live strain was capable of inducing interference at m.o.i. as low as 1 p.f.u./cell, whereas the inactivated virus induced it only at m.o.i. above 5–10 p.f.u./cell, regardless whether the interference was homologous or heterologous.

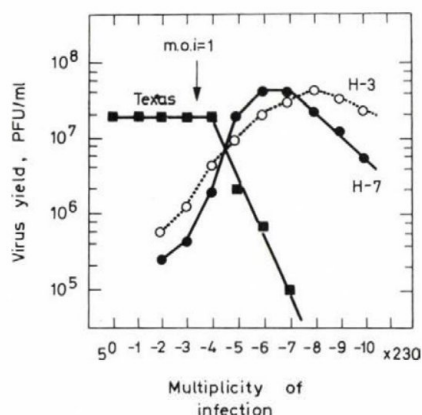


Fig. 2. Effect of m.o.i. on the yields of strains H and Texas. Virus stocks for inoculating CEF were prepared by infecting eggs with 10^3 (H-3) or 10^7 (H-7) p.f.u. of strain H

Auto-interference of the NDV strain H. It has been observed earlier that at a m.o.i. of about 10 p. f.u./cell, the growth of strain H is low as compared to the other plaque-forming strains [10, 15]. To obtain more information on this phenomenon, CEF cultures were infected with strain H at falling multiplicities and the virus yields were measured (Fig. 2). Two types of inoculum were used for infection of the monolayers; one had been harvested from eggs infected with 10^3 p.f.u. (H-3), the other from eggs infected with 10^7 p.f.u. (H-7).

The yields obtained with the two inocula were at a roughly uniform low level after infection at m.o.i. above 0.1 p.f.u./cell, but at multiplicities below that the yield increased about 100-fold. Cytopathic effect (c.p.e.) and IF-production were, however, different with the two kinds of inoculum (Fig. 3). Infection with H-7 was regularly followed by a moderate c.p.e., manifesting itself chiefly in a rounding-off of the cells, and a considerable amount of IF was induced, the amount of which fell parallel with the decrease of m.o.i. Infection with H-3 caused syncytium formation progressing to a complete

cell destruction at multiplicities above 0.1 p.f.u./cell and only a low amount of IF was induced. Thus, c.p.e. and IF-production seem to be related inversely.

The presence of 0.12 $\mu\text{g/ml}$ actinomycin D failed to alter the yield of strain H at multiplicities above 1 p.f.u./cell; it remained as low as without actinomycin (3×10^5 p.f.u./ml). Accordingly, inhibition of IF-production had no influence on auto-interference of strain H.

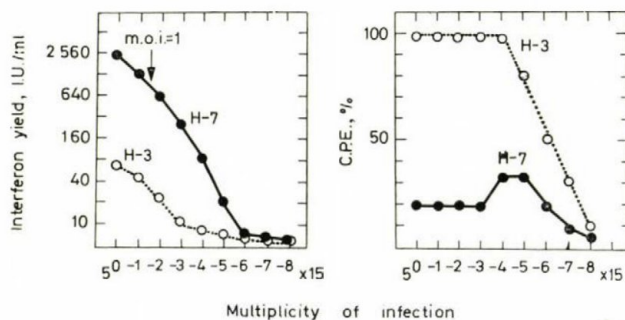


Fig. 3. Yield of IF and development of c.p.e. in CEF infected with different m.o.i. of H-3 and H-7. IF and CPE were measured at 23 hr after infection

Discussion

The role of IF was studied in homologous and heterologous interference elicited by certain NDV strain. In homologous interference, inhibition of growth of the velogenic NDV strain Texas, whereas in heterologous interference, inhibition of the yield of Sindbis virus was demonstrated. In contrast to BALUDA's [14] opinion that the main cause of virus inhibition is the suppression of adsorption, we found that homologous interference occurred not so much at the level of virus adsorption as after it [1-3] and that live virus was usually a more efficient growth inhibitor than inactivated virus. The role of IF in interference phenomena was clarified by means of specific metabolic inhibition by actinomycin D [16] and strains inducing and not inducing IF were compared for their capacity to interfere. It was found that IF had no part in homologous interference, that interference was not sensitive to actinomycin D, and that live viruses inhibited growth when added to the system even after superinfection, whereas inactivated virus was not able to do so, presumably due to a lack of growth ability, as suggested earlier by BRATT and RUBIN [4]. ORLOVA *et al.* [7] established that multiplication of the superinfecting virus decreased owing to a disturbance of its RNA synthesis. ROTT *et al.* [17] arrived at similar conclusions concerning influenza virus.

Heterologous interference of NDV was investigated by LEVINE [5] who observed the diminished multiplication of both NDV and WEE, without explaining the underlying mechanism. The present findings weigh in favour of the hypothesis that NDV inhibits the growth of Sindbis virus through the IF which it stimulates, because the IF-inducing NDV strains had a more pronounced inhibitory effect than non-inducing ones and this could be reversed with actinomycin D. The degree of interference still tended to increase after 2 hr. IF could act as a mediator of interference because the Sindbis virus is highly sensitive to IF. It follows that IF mediates virus interference only in those systems in which the IF-inducing virus encounters an IF-sensitive virus. In certain cases, inhibition of the IF-sensitive virus is, however, mediated by a factor other than IF, as *e.g.* in the system of ZEBOVITZ and BROWN [6]. Interference based on the IF-system was described in the absence of detectable amounts of IF [18].

Earlier studies along this line [19, 20] suggested that at high m.o.i. with NDV, the VON MAGNUS phenomenon described for influenza virus [21] did not take place in chicken cells. HERMODSSON [8], however, observed that autointerference of NDV in calf kidney cells was due to the IF stimulated during replication of the virus. In the present study, autointerference of the IF-inducing H strain in CEF cultures was found to be unrelated to IF, because actinomycin D failed to reverse it. It was regularly observed that independently of the decreased growth ability of the virus, high IF yields were associated with a moderate c.p.e. whereas low IF yields with complete cell destruction. Although the growth of NDV is less inhibited by IF than the growth of other viruses, NDV-induced c.p.e. is considerably decreased by IF-treatment (unpublished data), suggesting that the IF induced during viral replication is probably responsible for the mild c.p.e. On the other hand, it cannot be excluded that IF production of strain H was made possible by the milder c.p.e. [10]. It is noteworthy that SREEVALSAN and LOCKART [9] were able to exclude the mediator role of IF in the auto-interference of the IF-sensitive WEE. Preliminary studies [22] indicated that strain H was composed of a mixed population different in virulence, plaque morphology and growth ability, and that its behaviour was influenced by the proportion in the inoculum of slow and fast growing mutants.

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MICROBIOLOGICAL DEGRADATION OF DIAZEPAM

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Summary. In studying the transformation of diazepam (7-chloro-1-methyl-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one) by fungi isolated from soil, N₁-demethylation and cleavage of the diazepine ring were observed. Three metabolites: 7-chloro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one, 2-acetamido-2'-benzoyl-4'-chloroacetanilide and 2-acetamido-2'-benzoyl-4'-chloro-N-methylacetanilide were isolated and identified.

The metabolism of diazepam has been studied in animals and in man by several authors [1–5]. Demethylation at the 1-nitrogen, hydroxylation at the 3-carbon of the seven-membered ring and at the *p*-position of the 5-phenyl group were observed. GREENSPAN *et al.* [6] described a process for the microbiological modification of diazepam by *Pellicularia filamentosa* strains. They obtained N-demethylated and 3-hydroxylated products which were also observed in the metabolism of diazepam in animals, and in addition, N₄-oxide of diazepam and 6-chloro-4-phenyl-2(1H)-quinazolinone derivatives.

In the present study we have attempted to transform diazepam with fungi isolated by us from soil.

Materials and methods

Microorganisms. The fungi were isolated from natural habitats such as soil or tree trunk using the following method. A soil sample of 0.5–2 g was suspended in 100 ml aqueous solution of crystal violet (4 mg/100 ml) and was shaken at room temperature for 2 hr. The suspension was then allowed to stand and after 30 min the supernatant was mixed with an equal volume of aqueous 3% agar solution at 45°C and 1.5 ml of the mixture were placed onto 20 ml of a solid medium in Petri-dishes. This solid medium contained (per liter of tap water): peptone, 8 g; dextrose, 18 g; malt-extract, 8 g; corn-steep liquor, 2 g (dry weight); agar, 30 g; NaCl, 2 g; crystal violet, 20 mg; pH 5.2 (adjusted before sterilization).

The inoculated Petri-dishes were incubated at 26°C for 2 to 4 days. Crystal violet inhibited the growth of bacteria. The colonies of fungi were easily isolated from the dark blue agar plate and cultivated on potato dextrose agar. The isolates were classified by Dr. E. NOVÁK at the National Institute of Public Health, Budapest.

Fermentation conditions. Cultivation of the fungi and transformation of the substrate were carried out in 500 ml Erlenmeyer flasks containing 100 ml of the medium. Two different media were used.

Medium A contained (per liter of tap water): peptone, 8 g; dextrose, 18 g; malt-extract, 8 g; corn-steep liquor, 2 g (dry weight); NaCl, 2 g; pH 5.5 (adjusted before sterilization).

Medium B contained (per liter of tap water): dextrose, 20 g; corn-steep liquor, 10 g (dry weight); NaCl, 10 g.

After inoculation, the flasks were incubated at 28°C on a rotary shaker (300 rpm, 2 cm

amplitude). After 48 hr 50 mg of diazepam dissolved in 1 ml of methanol was added to each flask and the incubation was continued for 72 or 96 hr.

Analysis of products. 5 ml samples taken from the bioconversion beers were extracted with an equal volume of ethyl acetate. Analysis of the extracts was performed by thin layer chromatography on Kieselgel HF₂₅₄₊₃₆₆ with chloroform : ethanol (93 : 7). The spots of substrate and products on the plates were directly visible under UV light. The R_F values for substrate and products were, diazepam: 0.7; compound I: 0.45; compound II: 0.35; compound III: 0.42.

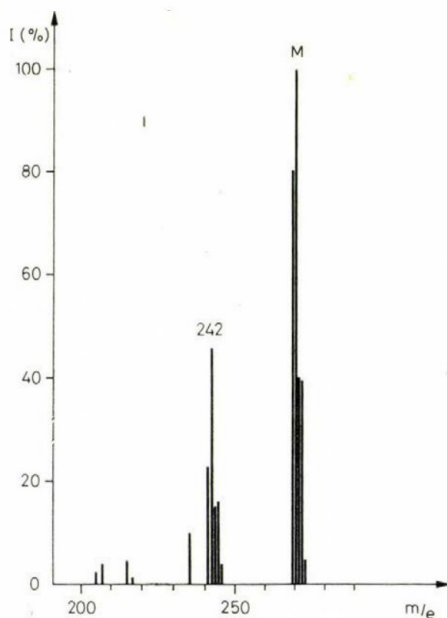


Fig. 1. Mass spectrum of compound I

For isolation of compounds I and II, 2.5 g of diazepam were distributed into 50 Erlenmeyer flasks containing *Aspergillus niger* J/8 culture grown on medium A. After incubation for 3 days the combined bioconversion beer was filtered and the filtrate was extracted three times with one third volume of ethyl acetate. The ethyl acetate extracts were evaporated under reduced pressure. The residue (3.16 g) was taken up in 100 ml mixture of methanol : water (85 : 15) and extracted twice with one third volume of petroleum ether. From the aqueous methanol phase the methanol was removed under reduced pressure and the remaining aqueous solution was extracted twice with an equal volume of ethyl acetate. The ethyl acetate extract was evaporated to dryness *in vacuo*. The residue containing compounds I and II as well as a small amount of diazepam was separated by thin layer chromatography on plates coated with a 1 mm thick layer of a 2 : 1 mixture of Kieselgel G and Kieselgel HF₂₅₄₊₃₆₆. The chromatograms were developed with a mixture of chloroform : ethanol (93 : 7). From zones with mobilities of compounds I and II, the adsorbent was scraped off and eluted with methanol. The methanol solutions were evaporated to dryness. The residue (330 mg) obtained from the eluate of compound I was recrystallized from ethanol yielding 180 mg of compound I (m.p. 214–216°C).

IR spectrum: ν_{NH} 3250–2700; amide-I 1680; $\gamma(=\text{CH})$ 795, 745; γ_{CC} 705 cm^{-1} . Mass spectrum of compound I: Fig. 1.

The residue (180 mg) obtained from the eluate of compound II was recrystallized from acetone yielding 105 mg of compound II (m.p. 136–139°C).

IR spectrum: ν_{NH} 3310; amide-I 1695–1660; $\nu_{\text{C=O}}$ (Ar) 1640; β_{NH} 1510; $\gamma(=\text{CH})$ 750; γ_{CC} 705 cm^{-1} . Mass spectrum of compound II: Fig. 2.

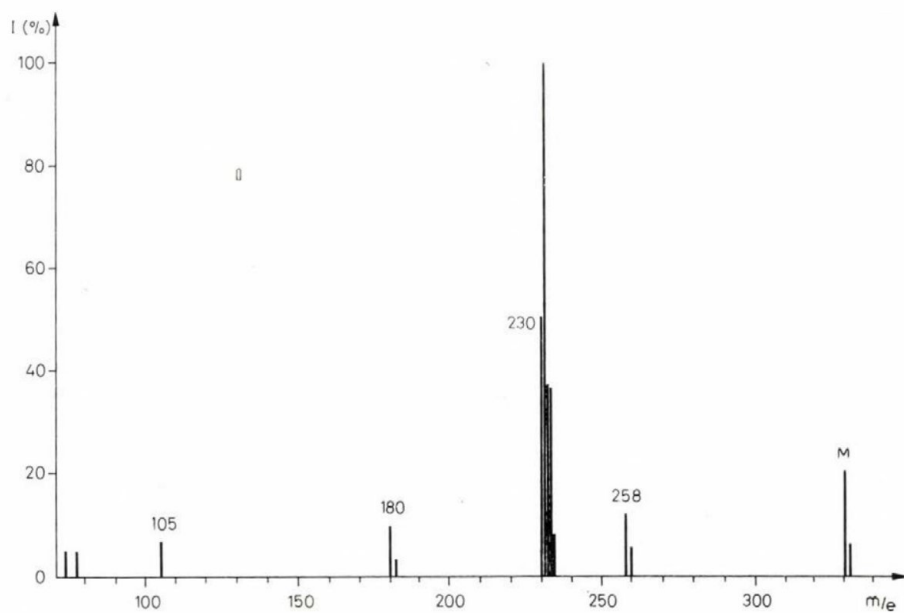


Fig. 2. Mass spectrum of compound II

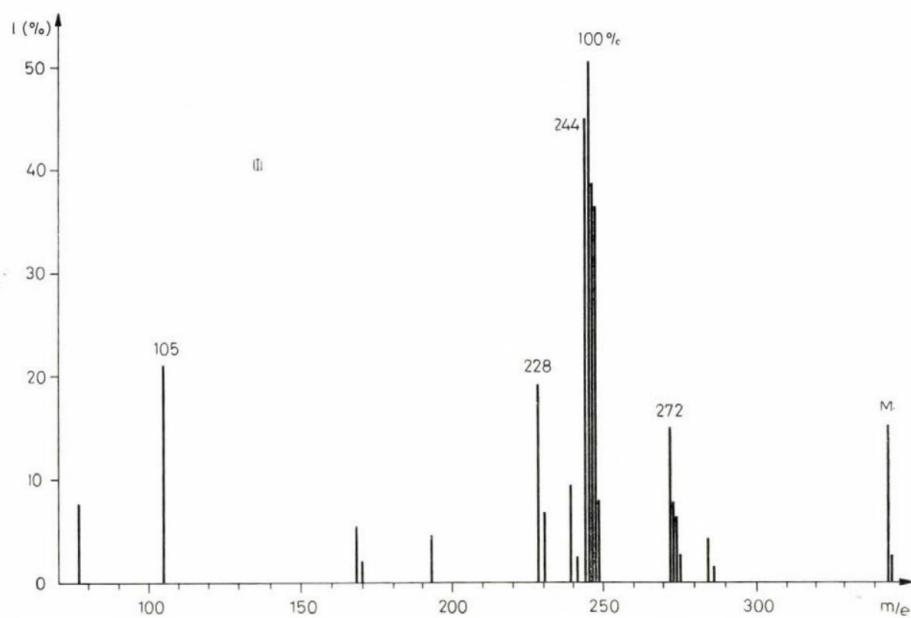


Fig. 3. Mass spectrum of compound III

For isolation of compound III, 2.5 g of diazepam were distributed into 50 Erlenmeyer flasks containing *Penicillium velutinum* culture grown on medium B. After incubation for 3 days diazepam was consumed and compound III accumulated in the fermentation beer. Compound III was extracted and purified by preparative thin layer chromatography as described above for compounds I and II. Crystallization from acetone yielded 420 mg of compound III (m.p. 107–110°C).

IR spectrum: ν_{NH} 3360; amide-I 1680–1660; $\nu_{\text{C=O}}$ (Ar) 1642; γ (=CH) 805, 750; γ_{CC} 708, 702 cm^{-1} . Mass spectrum of compound III: Fig. 3.

Acid hydrolysis. Fifty mg of compound I were heated with 50 ml of 4N HCl for 20 min on a steam bath. The cooled acidic solution was extracted with 50 ml of ether. The extract was washed with water, dried over Na_2SO_4 and evaporated to dryness. The residue was recrystallized from methanol affording 25 mg of compound IV (m.p. 95–96°C).

IR spectrum: ν_{asNH_2} 3410; ν_{sNH_2} 3310; $\nu_{\text{C=O}}$ + β_{NH} 1620–1580; γ (=CH) 765, γ_{CC} 705 cm^{-1} .

Compound IV was identified by comparison of its m.p., IR spectrum and thin layer chromatographic behaviour with a sample of synthetic 2-amino-5-chlorobenzophenone. The hydrolysis of compound II afforded, similarly to that of compound I, 2-amino-5-chlorobenzophenone.

Hydrolysis of 50 mg of compound III with the same method yielded 23 mg of compound V (m.p. 93°C).

IR spectrum: ν_{NH} 3350; $\nu_{\text{C=O}}$ 1628; β_{NH} 1560; γ (=CH) 755; γ_{CC} 702 cm^{-1} .

Compound V was identified by comparison of its m.p., IR spectrum and thin layer chromatographic behaviour with a sample of 2-methylamino-5-chlorobenzophenone obtained from diazepam by acidic hydrolysis [7].

Thin layer chromatography of the hydrolysis products was performed on Kieselgel HF₂₅₄₊₃₆₆ plates with heptane : ethyl acetate (90 : 10). Compounds IV and V after chromatography were directly visible as yellow spots. R_F values: compound IV, 0.15; compound V, 0.32.

Equipment. Melting points were measured with a micro apparatus (Franz Küstner Nachf. K. G. Dresden). Infrared spectra were determined in KBr pellets with a Perkin Elmer model 457 spectrometer.

Mass spectra were taken on a Varian Mat SM-1 mass spectrometer. Operating conditions: resolution, 1500; accelerating voltage, 8 kV; electron energy, 70 eV; source temperature, 250°C; evaporation temperatures: compound I, 155°C; compound II, 150°C and compound III, 90°C. High resolution mass measurements were carried out at $R = 10\,000$, using PFK as reference standard.

Results and Discussion

Studying the transformation of diazepam by fungi, 172 isolates obtained from natural habitats were tested. The majority of the strains did not metabolize diazepam. The strains shown in Table I accumulated metabolites from diazepam in significant amounts according to the analysis performed by thin layer chromatography of extracts of the bioconversion beers.

Compound I was found to be identical with the N-demethylated derivative of diazepam, 7-chloro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one on the basis of its m.p., IR spectrum and mass spectrum [8, 9]. Hydrolysis of compound I with HCl afforded 2-amino-5-chlorobenzophenone in accordance with the literature [1].

On the basis of its mass spectrum, compound II was supposed to be 2-acetamido-2'-benzoyl-4'-chloroacetanilide. For comparison we synthesized this compound from 2-amino-5-chlorobenzophenone, as described [10, 11]. M.p. and IR spectrum of the metabolite were identical with those of the synthetic compound.

Table I

Formation of metabolites from diazepam by different fungi

Fungi	Metabolites of diazepam		
	compound I	compound II	compound III
<i>Aspergillus niger</i> J/8	+	+	
<i>Aspergillus niger</i>	+		
<i>Aspergillus japonicus</i>	+	+	
<i>Circinella sydowi</i>	+		
<i>Myrothecium roridum</i>	+		
<i>Penicillium adametzi</i>	+		
<i>Penicillium velutinum</i>			+
<i>Rhizopus arrhizus</i>	+		

Judging from the similarity of the mass spectra of compounds II and III, we assumed that compound III was an N-methyl derivative of compound II.

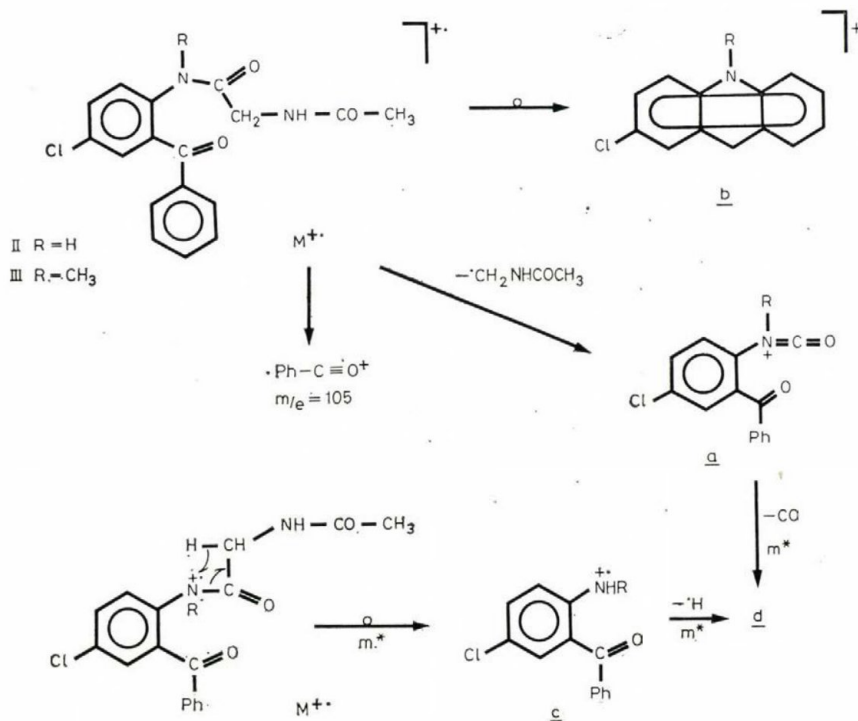


Fig. 4. Proposed mass-spectral fragmentation scheme of compounds II and III

Table II

High resolution mass measurements of ions shown in Fig. 4

Symbol	Compound	m/e	Mass decimals			Elemental composition	Difference (ppm)
			measured	measured — $^{35}\text{Cl}^*$	calculated for		
M	II	330	075 756	106 896	108 27	$\text{C}_{17}\text{H}_{13}\text{N}_2\text{O}_3(\text{Cl})$	4.1
a	II	258	032 086	063 226	06 333	$\text{C}_{14}\text{H}_9\text{NO}_2(\text{Cl})$	0.4
b	III	228	058 548	089 688	08 915	$\text{C}_{14}\text{H}_{11}\text{N}(\text{Cl})$	2.3
c	II	231	045 008	076 148	07 624	$\text{C}_{13}\text{H}_{10}\text{NO}(\text{Cl})$	0.4
d	II	230	037 726	068 866	06 841	$\text{C}_{13}\text{H}_9\text{NO}(\text{Cl})$	2.0

* Mass of ^{35}Cl used was 34.96886

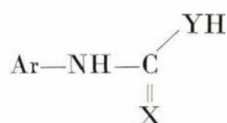
The fragmentation pattern of compounds II and III is demonstrated in Fig. 4.

The benzoyl-ion $m/e = 105$ generally appears in the spectrum of various benzophenones containing an unsubstituted phenyl group [12].

Ion *a* is formed by α -fission at the anilide carbonyl group.

A series of difficult rearrangements leads to the formation of ion *b*, to which the acridine structure shown was ascribed [9].

Base peak of the spectra of both II and III arises by a four-centre rearrangement, occurring in various types of compounds containing



grouping, e.g. aromatic amides [13, 14], aromatic ureas [15] and thioureas [16]. Formation of ion *c* via this process is indicated by metastable peaks at $m^* = 162$ –163 and at $m^* = 174$ –176 in the spectrum of II and III, respectively. Further loss of $\cdot\text{H}$, a typical fragmentation of anilines [17], yields ion *d* as shown by the appearance of metastable peaks at $m^* = 229$ –231 and at $m^* = 243$ –245 in the spectrum of II and III, respectively. Another way leading to ion *d* can be the loss of CO from ion *a*, as indicated by a metastable ion at $m^* = 220$ in the spectrum of III.

High resolution mass measurements of the ions depicted in Fig. 4 are summarized in Table II.

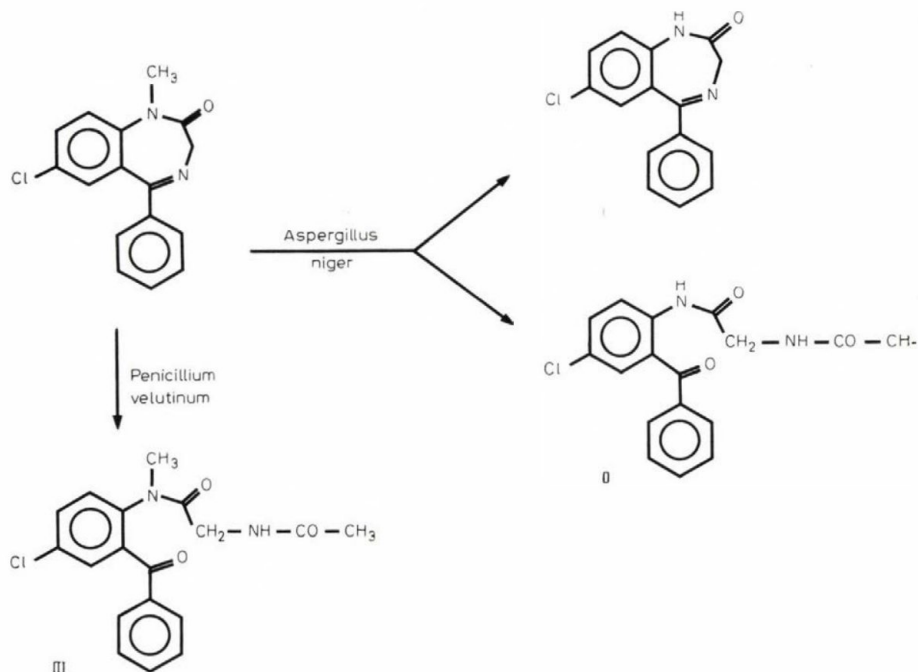


Fig. 5. Transformation of diazepam by *Aspergillus niger* J/8 and *Penicillium velutinum*

The structures ordered to compounds II and III were supported by the results of their hydrolysis. Hydrolysis of compound II by HCl resulted in the formation of 2-amino-5-chlorobenzophenone, while the hydrolysis of compound III afforded 2-methylamino-5-chlorobenzophenone. The latter hydrolysis product was also obtained from diazepam.

Our results show that N-demethylation of diazepam, reported by GREENSPAN *et al.*, [6] with *Pellicularia filamentosa*, occurs in the case of several fungi. The microbial cleavage of the seven-membered ring of diazepam resulting in the formation of N-acetylated metabolites (shown in Fig. 5) was first observed in the present study.

Acknowledgement. We are indebted to Dr. Zs. MÉHESFALVI-VAJNA for recording infrared spectra.

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TRANSFER OF *E1* AND *V* COLICINOGENIC FACTORS TO *SHIGELLA FLEXNERI*

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Summary. From *Escherichia coli* strains, ColE1-ML and ColV-K94 factors were transferred to a virulent *Shigella flexneri* 2a strain at 1–2% frequency. *S. flexneri* ColV⁺ and ColE1⁺V⁺ produced Col[–] segregants. The ColE1 factor was stably incorporated into *S. flexneri*. The ColV⁺ culture lost its virulence to the guinea pig eye.

In contrast to *Shigella sonnei*, *Shigella flexneri* produces colicins only rarely. The frequency in this group of colicinogenic strains is not more than 0–8.9% [1]. In recent years, 25 (5.9%) out of 419 *S. flexneri* strains isolated in Nógrád county have been colicinogenic. The reason for the unfrequent occurrence of colicinogeny in *S. flexneri* is not clear. In the present work, the behaviour of *V* and *E1* factors in, and their effect on, the recipient organism have been studied.

Materials and methods

Strains. A freeze-dried culture of strain *S. flexneri* 2aV was received from Professor I. KÉTYI, Pécs. The organism had been isolated from a virulent clone, as indicated by "V". It produced highly refractive, homogeneous, convex colonies and is characterized by antigenic formula II (3, 4). The strain is resistant to both *E1* and *V* colicins. The donor strains and crosses performed are shown in Table I. Colicin production was detected with the use of a method and indicator strains described previously [2].

Culture media. Complete solid and liquid media described in reference [2] were used. Selective isolation of *S. flexneri* from the mixed suspensions was carried out on desoxycholate citrate (DC) agar.

Transfer of colicin factors to *S. flexneri* 2a. Overnight cultures of *E. coli* (1 ml) and *S. flexneri* (2 ml) were mixed in a 100 ml Erlenmeyer flask then 7 ml fresh medium was added. After incubation at 37°C for 24 hr the culture was streaked on DC medium. Next day duplicate subcultures were made from 100 lactose negative colonies on complete medium (10 cultures on one plate). After incubation overnight, one of the duplicate cultures was killed with chloroform and overlaid with the indicator culture. Duplicates of Col⁺ colonies (exerting an inhibitory action) were checked by agglutination and biochemical test. Col⁺ *S. flexneri* strains reisolated in this manner and *S. flexneri* cultures from the control crosses were subcultured on agar slants and examined in parallel experiments.

Virulence was tested by SERÉNY's guinea pig eye test [3].

Results

Transfer frequency. The transfer from *E. coli* donors to *S. flexneri* 2a was successful for ColV in 2%, for ColE1 in 2%, for ColE1 + V in 1%. Cultures reisolated from the crosses corresponded in colony morphology and antigenic

structure to the parent *S. flexneri* 2a strain. The sugar fermentation spectrum remained the same, but the indole producing capacity of the parent strain was lost in all derivatives including those cultured from the control crosses.

Stability of Col⁺ character were suspended in saline and streaked on solid complete medium. Next day 100 isolated colonies were examined for colicinogeny.

S. flexneri 2aV⁺ split off in the first subculture 10% *Col⁻* colonies. Further subcultures of *Col⁻* colonies failed to produce *Col⁻* derivatives. Subsequent transfers of *Col⁺* colonies onto solid medium resulted in a 10–15% loss of the *ColV* factor.

Table I

E. coli × *S. flexneri* crosses

<i>E. coli</i> Hfr Cavalli* × <i>S. flexneri</i> 2a
<i>E. coli</i> F-PA309 (λ)* × <i>S. flexneri</i> 2a
<i>E. coli</i> F-PA309 (λ) (V-K94) × <i>S. flexneri</i> 2a
<i>E. coli</i> F-PA309 (λ) (E1-ML) (V-K94) × <i>S. flexneri</i> 2a
<i>E. coli</i> Hfr Cavalli (E1-ML) × <i>S. flexneri</i> 2a

* Control strains

Derivative *S. flexneri* 2a *El⁺V⁺* showed a similar *Col⁻* segregation, with the difference that in addition to the 10–20% *Col⁻* frequency, approximately 10% of the colonies exhibited a “transient” behaviour between *Col⁺* and *Col⁻* (the zone of inhibition was narrower than with *Col⁺* colonies). The *ColV* and *ColEI* factors were always lost simultaneously. If *ColV⁺* and *ColEI⁺V⁺* *S. flexneri* were cultured in liquid medium, the cells remained colicinogenic in 100%.

Derivative *S. flexneri* 2a *El⁺* retained its colicinogeny after serial passages.

Effect of Col factors on the virulence of S. flexneri 2a. One loopful of a definitely *Col⁺* colony gave rise to keratoconjunctivitis 1–2 days later than the control culture. From eyes inoculated with *ColV⁺* and *ColEI⁺V⁺* cultures, only *Col⁻* organisms could be recovered after 48 hr, either from cotton swab specimens or from the homogenate of the enucleated eye. In order to make the experiments more comparable, each of the *Col⁺* and the control *S. flexneri* broth cultures of identical OD (2×10^9 cells/ml) was inoculated with standard loops into 5 guinea pig eyes. Animals inoculated with *ColV⁺* and *ColEI⁺V⁺* organisms developed no keratoconjunctivitis; *ColEI⁺* and the control cultures produced typical keratoconjunctivitis in 24–36 hr. Swab specimens from eyes that had been inoculated with liquid *ColV⁺* and *ColEI⁺V⁺* cultures, failed to show the presence of shigellae, but those infected with *ColEI⁺* and

control organisms yielded positive results. *Col*⁻ segregants obtained on sub-culturing, produced a keratoconjunctivitis similar to that obtained with the parent and control cultures. The results are summarized in Table II.

Table II
Effect of Col factors on experimental keratoconjunctivitis

Strain	Virulence	Colicinogeny of cultures recovered from the eyes, per cent	Incorporation of Col factors into <i>S. flexneri</i>
<i>S. flexneri</i> 2a (control strains)	Virulent	.	.
<i>S. flexneri</i> 2a <i>ColV</i> ⁺	Avirulent	0	Unstable
<i>S. flexneri</i> 2a <i>ColE1</i> ⁺ <i>V</i> ⁺	Avirulent	0	Unstable
<i>S. flexneri</i> 2a <i>ColE1</i> ⁺	Virulent	100	Stable

Discussion

STREELMAN et al. [4] observed that colicin modified the course of guinea pig keratoconjunctivitis. They showed in quantitative experiments that killing of the bacteria by colicin mitigated the infection and that apathogenic *S. sonnei* failed to survive in the guinea pig eye.

In our experiments, *S. flexneri ColV*⁺ and *ColE1*⁺*V*⁺ cultures lost their virulence upon the effect of *Col* factors, but *ColE1*⁺ derivatives gave rise to keratoconjunctivitis. Colicinogeny is a stable property of the bacterial cell. Colicin factors of *E. coli* are incompletely incorporated into *S. flexneri*, an alien host.

Similar findings were described by KÉTYI [5] concerning the transfer of *ColE1-a* and *ColB₂-drd* factors to *S. flexneri*; it was shown that the cultures produced no colicin after a few weeks, although *Col*⁺ colonies could be detected in the population.

Earlier experiments [6] indicated that cultures transferred to *Col*⁺ are similar to the parent organism in all characters except colicinogeny. In our experiments the influence of *Col* factor upon virulence may be explained by a decreased capacity for survival in the guinea pig eye of the *Col*⁺ derivatives. The rapid disappearance of these organisms is not associated with their sensitivity to lysozyme, but probably with an unknown cellular mechanism. The low frequency of intergeneric *Col* factor transfer, the instability of colicinogenic strains and the decreased survival of *Col*⁺ cells may be responsible for the rare occurrence of colicinogenic *S. flexneri* in nature.

Acknowledgement. The author is indebted to Professor I. KÉTYI for the *S. flexneri* strain.

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GENETIC AND BIOCHEMICAL ANALYSIS OF HAEMIN DEPENDENT MUTANTS OF BACILLUS SUBTILIS

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Summary. Numerous porphyrin auxotrophic mutants have been isolated from the 168 *trpC2* strain of *Bacillus subtilis* by selection with streptomycin. Some of them could be supplemented with ALA while the majority grew only in the presence of haemin. Among the latter strains, the syntropism test allowed to distinguish two groups different in phenotype, viz., feeders accumulating ALA and non-feeders accumulating instead of ALA other porphyrin intermediates. On the basis of transductional studies, feeders and non-feeders could be divided into two and four groups, respectively. Biochemical investigation revealed that, with one exception, one enzyme of the porphyrin biosynthesis was coordinated to each hem locus. The following genes were identified: *hemB* → ALA-dehydrase; *hemC* → PBG-deaminase; *hemE* → uroporphyrinogen decarboxylase; *hemF* → coproporphyrinogen oxidase; *hemG* → protoporphyrin-iron-chelatase.

Haemin dependent mutants can be isolated from the 168 *trpC2* strain of *Bacillus subtilis* by different methods [1]. Selection on plates containing streptomycin was found to be the method most effective for the isolation of haemin auxotrophs [2]. Some of the strains deficient in porphyrin biosynthesis can grow in the presence of δ -amino-laevulinic acid (ALA) and probably have a defect of ALA-synthetase; the requirement of the majority of auxotrophs can be met only with haemin.

In the present study, a number of haemin requiring mutants have been isolated by selection with streptomycin [2] and their genetical and biochemical characterization has been attempted. The aim of these studies was to reveal any genetical heterogeneity among the haemin auxotrophs and to determine the number of the different *hem* loci in the isolated auxotrophs. The biochemical studies have been carried out to determine the enzyme deficiencies of the auxotrophs.

Materials and methods

Strains. Haemin dependent auxotrophs were isolated from *B. subtilis* strain 168 *trpC2*. Twenty-one of the one-hundred and ninety-eight haemin dependent isolates proved to be ALA auxotrophs. The strain *hemA1* was used as reference strain [3].

Media. A yeast extract peptone medium (YP) was used routinely as complete medium [4]. The chemically defined medium designated GGM [1] was supplemented with 50 μ g/ml tryptophan and 2 μ g/ml ALA was added for *hemA1* mutants. HCA medium [2] was used for cultivating haemin auxotrophs; it consisted of GGM medium supplemented with 50 μ g tryptophan, 2.5 μ g haemin, 50 μ g cysteine and 0.5 mg bovine albumin (Calbiochem) per ml of medium. YPB (blood extract medium): citrated ox blood was washed in saline and the red blood cells

were suspended in YP liquid medium at 10% (v/v) final concentration. The suspension was heated to 100°C, the precipitate removed by centrifugation under aseptic conditions and the clear supernatant was heated at 80°C for 10 min on two subsequent days. Solid media were prepared with 1.5% agar.

Transduction. Transduction was performed with 3NT subtilis phage as described earlier [3]. Selective media were supplemented according to the auxotrophic requirements in HCA medium under aeration until OD 0.4 had been reached (Bausch Lomb Spectron 20, 620 nm, 2×10^8 colony forming units/ml), then the suspension was infected at a multiplicity of 1 and left at room temperature for 30 min. The bacteria were sedimented by centrifuging at 2000 rpm, washed in GGM, the pellet was resuspended in the original volume of GGM, then 0.1 ml aliquots were plated on the selective media. At least five plates were made of each sample. The number of spontaneous revertants was subtracted from the number of transductants.

Determination of ALA-dehydrase (E.C. 4.2.1.24) activity. The method of JAYARAMAN *et al.* [5] was followed with some modifications. Bacteria were grown in YPB liquid medium at 37°C under continuous shaking. After reaching OD 0.8–0.9, the culture was centrifuged and 1 g of bacteria (wet weight) was resuspended in 5 ml of 0.05 M glycine-NaOH buffer (pH 9.2). The suspension was chilled, sonicated (MSE Ultrasonic Desintegrator Model 150 W at 12 μ amplitude) and centrifuged again in the cold. Ten μ mol ALA (in 0.1) as substrate and 0.9 ml 120 μ mol glycine-NaOH buffer were added to 1 ml of the supernatant. The reference lacking the substrate as well as the samples were incubated at 55°C for 1 hr, then 1 ml TCA-HgCl₂ solution (5% TCA, 0.02 M HgCl₂) was added to the assay systems. The precipitate was removed and 2.5 ml samples were taken, and an equal amount of modified Ehrlich's reagent was added [6]. The porphobilinogen (PBG) formed after standing for 5 min was determined by measuring absorbance at 555 nm in a Beckman DU spectrophotometer. The reference curve was constructed with an authentic sample of PBG. Unit ALA-dehydrase activity was defined as 10 nmol PBG formed by 1 mg protein during 1 hr. Protein was determined according to LOWRY *et al.* [7].

Accumulation of PBG. This was determined in the protein-free extract of the auxotrophic strains as described above.

Detection of porphyrin synthesis in auxotrophic cells. In 1 liter Erlenmeyer flask with 200 ml of YP medium supplemented with 2.5 μ g/ml haemin, 50 μ g/ml cysteine and 0.5 mg/ml bovine albumin were inoculated with the haemin auxotroph under investigation. The culture was aerated under shaking at 250 rpm (Gyratory Incubator Shaker) at 37°C for 20 hr. By this time the optical density value was approximately 1. The cultures were filled up to 800 ml with YP medium, distributed again into 200 ml parts and shaken for additional 12 hr. This manipulation was necessary to dilute out the haemin which had not been used up in order to prevent a feed-back inhibition of haem synthesis. The cultures were centrifuged and the bacterial pellet as well as the supernatant were treated separately as follows.

Concentration of extracellular porphyrins: 10 ml of 10 N HCl and 10 g talcum were added to 1 litre of the supernatant. After shaking several times during a two-hour period, the talcum was removed by filtering through a glass sintered G₃ filter (Zeiss, Jena), and washed with distilled water. The adsorbed porphyrins were eluted from the talcum with 10 ml of an acetone-10 N HCl mixture (9 : 1, v/v). The solution was evaporated in a "rotavapor" under vacuum at 45°C in the dark. The dry material was stored in the refrigerator for less than 48 hr. Then the material was re-dissolved in 0.6 ml of the acetone-HCl mixture as described above and used within 2 hr. Samples of 50 μ l were taken and chromatographed on Whatman's No. 1 paper.

Extraction of intracellular porphyrins: 1 g of bacteria (wet weight) was extracted under shaking in 5 ml acetone-HCl at 4°C for 4 hr [8]. The extract was centrifuged, evaporated, stored and re-dissolved as above.

Chromatography. To avoid the disturbances caused by chromogenes in concentrates, two-dimensional ascending chromatography was used. The size of the paper was 20 $\times$ 20 cm, the solvents were 0.1 M LiCl in the first [9], and 2.6 lutidine : water (10 : 4, v/v) in the second dimension [10]; both were carried out in ammonia-saturated atmosphere. The spots fluorescing under UV-light (through a Wood filter) were cut out and extracted overnight in 0.5 ml of 1.5 N HCl. The chromogen-free porphyrin fractions were re-chromatographed in the solvents mentioned above and their R_F value was determined. Uroporphyrin I octamethyl-ester, coproporphyrin I and III tetramethyl-ester as well as protoporphyrin IX dimethyl-ester (Calbiochem) were hydrolyzed [11] and used for references.

Quantitative determination of porphyrins. The spots cut out from the two-dimensional chromatograms were extracted in 2 ml 1.5 N HCl each. Quantitative determination of porphyrins was made on the basis of their fluorescence. Calibration curves were made with uroporphyrin I, coproporphyrin III and protoporphyrin IX standards. Excitation and measurement

of the emission were done at 402 and 595 nm (uroporphyrin), 400 and 595 nm (coproporphyrin), 404 and 600 nm (protoporphyrin), in an Opton PMQ II spectrophotometer (position I). Slides were 1.6 for the exciting light and 1.8 for the emission at a fortification of 10. The concentration optimum for measurements was between 0.05–0.5 $\mu\text{g/ml}$ under such circumstances. Quantitative values were given in μg porphyrin/g bacteria (wet weight). The 3-COOH intermediary was estimated at the wavelength used for coproporphyrin.

Results

Grouping of the mutants by syntropism. ALA auxotroph designated *hemA1* [1] was used as indicator. A linear inoculation was made on a YP agar plate supplemented with 0.5 $\mu\text{g/ml}$ haemin which supported suboptimal growth and a few auxotrophs to be tested were inoculated near to the indicator bacteria. In this way, 177 auxotrophs requiring haemin were tested and divided into two groups. The majority of the strains (111 isolates) showed the syntropism phenomenon in 48 hr; this indicated an accumulation of ALA. These isolates will be referred to below as "feeder". The rest of the strains (66 isolates) did not show any syntropism. They will be referred to as "non-feeder".

Grouping of the auxotrophs by recombination. Phage 3NT was propagated on two feeder and two non-feeder strains selected in preliminary tests. The

Table I

Preliminary grouping of 17 haemin requiring mutants and their transduction to haemin independence by phages propagated on selected donors

Recipients			No. of haemin independent transductants by the indicated donors			
Groups of mutants	Designation of mutants	Phenotype	Feeders		Non-feeders	
			614	9	29	234
I	1, 9, 101, 117, 270, 272, 375, 393	feeders	0.9 ^a	0.0	28.1	44.3
			(0–2) ^b	(0)	(15–55)	(13–85)
II	33, 48	feeders	4.4	8.1	35.8	50.5
			(4–5)	(7–9)	(32–42)	(48–54)
III	321, 339, 722	non-feeders	58.2	130.1	0.6	41.2
			(20–124)	(68–168)	(0–1)	(24–54)
IV	234, 338, 344, 367	non-feeders	54.1	111.5	39.6	0.6
			(30–73)	(78–140)	(25–79)	(0–2)

^a average per plate of transductants of all strains belonging to the same group

^b highest and lowest values for transductants obtained on individual plates with various strains belonging to the same group

Table II

Grouping of haemin requiring mutants and their transduction to haemin independence by phages propagated on No. 321 and No. 344 isolates as donors

Groups of mutants	Recipients		No. of haemin independent transductants by the indicated donors	
	Designation of mutants	Phenotype	321	344
III	15, 23, 29, 32, 36, 40, 58, 61, 106, 112, 119, 146, 152, 161, 188, 208, 217, 236, 245, 248, 265, 291, 311, 318, 372, 380, 381, 603, 621, 622, 624, 632	non-feeders	0.1 (0-1)	61.1 (41-101)
IV	107, 173	non-feeders	32.5 (28-37)	0.1 (0-1)
V	64, 109, 127, 130, 271, 357, 627	non-feeders	2.7 (2-4)	39.0 (21-58)
VI	180, 214, 601, 606	non-feeders	16.0 (11-34)	26.6 (15-44)

Table III

Examination by transduction of genetic homogeneity of groups V and VI

Groups of mutants	Recipients		No. of haemin independent transductants by the indicated donors	
	Designation of mutants	Phenotype	64	180
V	64, 109, 127, 130, 271, 357, 627	non-feeders	0.1 (0-1)	35 (24-62)
VI	180, 214, 601, 606	non-feeders	28 (19-54)	0.1 (0-1)

lysates were sterilized by filtration and transduction test made on 17 other auxotrophs. Since the transductional effectivity of phage 3NT varies in the different lots [4, 12], the same phage preparations were used in the transductional experiments. The Tables show the average number of transductants per plates of all the strains belonging to the same group. The numbers in brackets show the lowest and the highest values for the transductants obtained on individual plates.

Table IV

*Cotransduction of haemin independence with hemA1 marker
(ALA requirement) by phage propagated on hemA1*

Group of recipient mutants	Genotype of the transductants		Cotransduction of the markers, per cent
	hem ⁺ ALA ⁺	hem ⁺ ALA ⁻	
I 1, 9, 101, 117	245	2503	91
II 33, 48	58	411	87
III 321, 339, 722	1219	27	2
IV 234, 338, 344, 367	2053	0	0
V 64, 109, 127	521	0	0
VI 180, 214, 606	315	622	50

Table V

*Specific activity of ALA-dehydrase in haemin requiring
mutants grouped previously by transduction
(Specific activities in the parent strain and hemA1 are given below)*

Group	Designation of strains	Specific activity of ALA-dehydrase, units	Group	Designation of strains	Specific activity of ALA-dehydrase, units
I (feeder)	1	0	III (non-feeder)	321	67.6
	9	0		339	102.1
	117	0		722	83.4
	101	0	IV (non-feeder)	234	27.4
	270	0		338	40.8
	272	0		344	30.5
	375	0		367	29.0
	393	0			
II (feeder)	33	16.6			
	48	25.3			

Bacillus subtilis strain 168 *trpC2* 41.5

hemA1 20.5

According to the results, the 17 recipients tested could be distributed into four groups (Table I). Crosses were made between strains of feeder and non-feeder phenotypes; if the number of hem⁺ transductants was high, this

Table VI
Amount of PBG accumulated by the strains
of the groups studied*

Groups	Designation of strains	PBG nmol
	<i>B. subtilis trpC2</i>	0
	<i>hemA1</i>	0**
I	1, 9	0**
II	33	62.5
	48	45.0
III	321	0
IV	344	0

* The values refer to the amount of PBG in 1 g bacteria (wet weight)

** Strains of group I have no ALA-dehydrase activity while *hemA1* lacks ALA-synthetase

Table VII
 R_F values of the isolated porphyrin intermediates in ascending paper chromatography

Strains tested	R_F values of the accumulated porphyrins							
	uroporphyrin		copro III		3-COOH		proto IX	
	1	2	1	2	1	2	1	2
<i>B. subtilis trpC2</i>	—	—	0.28	0.24	—	—	—	—
<i>hemA1</i>	—	—	—	—	—	—	—	—
I/1	—	—	—	—	—	—	—	—
II/33	—	—	—	—	—	—	—	—
III/321	0.91	0.0	0.28	0.24	0.20	0.53	0.03	0.68
IV/344	—	—	0.28	0.24	—	—	—	—
V/64	0.91	0.0	—	—	—	—	—	—
VI/180	0.91	0.0	0.28	0.24	0.20	0.53	—	—
standards	0.90	0.0	0.29	0.23	0.20*	0.54*	0.02	0.69

— no porphyrins detectable in the extract;

* R_F values according to WITH [9] and ERIKSEN [10]

Solvents: 1 = 0.1 M LiCl (NH₃)

2 = 2,6-lutidine : water (10 : 4, NH₃)

indicated that their *hem* loci were far apart. When the site of the markers was the same in the donor and the recipient, no transductants were found.

Further transductional tests were made with 45 non-feeder isolates which had not been included in the former experiments. In this case transducing phage was prepared on strains 321 and 344 of groups III and IV, respectively. The results of these experiments are shown in Table II. The majority of

Table VIII

Quantitative determination of the isolated porphyrin intermediates according to fluorescence

Strains tested	uroporphyrin		copro III		3-COOH		proto IX	
	intra-	extra-	intra-	extra-	intra-	extra-	intra-	extra-
	cellular		cellular		cellular		cellular	
<i>B. subtilis trpC2</i>	0*	0	0.54	0.96	0	0	0	0
III/321	0	2.61	0.44	20.49	0.76	0.28	0	19.13
IV/344	0	0	0.95	2.36	0	0	0	0
V/64	3.0	18.9	0	0	0	0	0	0
VI/180	0	3.1	0.48	52.2	0	1.9	0	0

The values refer to μg porphyrin/g bacteria (wet weight)

* No detectable amount of the metabolite was found

the strains tested belonged to group III and two strains to group IV. However, group V and group VI had to be created as well for the strains which were different from any of the former ones. It can be seen in Table II that the *hem* loci of groups III and V are near each other because only a small number of *hem*⁺ transductants resulted from their crosses. To test the homogeneity of groups V and VI, phage 3NT was propagated on strains V/64 and VI/180 (the group and the strain are denoted by Roman and Arabic numerals, respectively) and transductions were made with them on all the strains belonging to groups V and VI (Table III). According to the experiments made with homologous phage preparations, the recipients tested did not yield any *hem*⁺ recombinants. A possible co-transduction of the *hemA1* locus with the *hem* markers of the different mutants belonging to the six groups was also tested. In these experiments, haemin requiring mutants were treated with phage propagated on the *hemA1* donor strains and the bacteria were plated onto GGM + 2 $\mu\text{g}/\text{ml}$ ALA medium. The ratio of recombinants was determined by replicating the transductant colonies onto GGM without ALA. When calculating linkage, the number of transductants obtained with strains belonging to the same group were added. As it can be seen in Table IV, the *hem* loci of the strains of different groups are linked to the *hemA1* locus either to different extents or not at all.

Biochemical study of the mutants. Detection of ALA-dehydrase (EC 4.2 1.24.) was performed with strains belonging to groups I and II. The parent strain, *hemA1*, as well as several other strains belonging to groups III and IV were also tested as controls. Table V summarizes the specific enzyme activities of the cell extracts. Only strains of group I had no enzyme activity and so were deficient in ALA-dehydrase.

On the basis of their PBG accumulation, strains of group II (Table VI) could sharply be distinguished from all the other strains. In the case of the parent strain as well as of those of groups III and IV, the lack of PBG accumulation is explained by a further metabolism of the compound.

Chromatographic examination of porphyrins. Qualitative determinations of the extracellular porphyrins were made in the case of the parent strain, *hemA1*, and a representative strain of each group. All the strains examined were grown in YP medium containing haemin and the necessary supplements. Tests made with strains *hemA1*, I/1 and II/33 were expected to confirm the former results, i.e. not to accumulate or synthesize porphyrins. Table VII summarizes the intermediate compounds excreted and their R_F values. As a 3-COOH standard was not available, it was identified merely by its R_F value given by references.

In the case of strain II/33, no direct test of enzyme activity was made except the detection of PBG accumulation. No tetraporphyrin intermediates could, however, be detected chromatographically and therefore it seemed reasonable to assume that PBG-deaminase was defective in strain II/33. The conversion of PBG into uroporphyrinogen III is known to be mediated by two enzymes [13, 14], PBG-deaminase and PBG-isomerase; with the latter functioning only if the PBG-deaminase is active. In the case of a defective PBG-isomerase, only intermediates of the porphyrin I isomer series could be detected.

Quantitative determination of porphyrins. Absorption and emission spectra were determined in 1.5 N HCl and the measurements were made at the maxima. Table VIII shows the quantities of accumulated porphyrins. The values given are averages of two independent determinations. Strains of group III accumulated coproporphyrin III and a small amount of uroporphyrin in addition to protoporphyrin IX. In this case, the defect was probably in the ferrochelatase enzyme. It was surprising that strain IV/344 which could grow only in a medium containing haemin, should have accumulated the same intermediate in nearly the same amount as the parent strain did. Group V accumulated only uroporphyrin; a defective uroporphyrinogen-decarboxylase was assumed in this case. Group VI accumulated uroporphyrin, a large amount of coproporphyrin III and a small amount of the 3-COOH intermediate but no protoporphyrin IX; accordingly a defect in coproporphyrinogen-oxidase could be assumed here.

Discussion

Defects of haem biosynthesis cause lethality in the case of the obligate aerobic *B. subtilis* because haem is a prosthetic group of cytochromes and of catalase. The study of porphyrin auxotrophs of this organism is difficult as they can utilize only the first (ALA) and the last (haem) products from their environment. As intermediates, they cannot utilize uroporphyrin and coproporphyrin; probably because the reduced forms, the porphyrinogens, are oxidized spontaneously in the system. Haem auxotrophs were also unable to

Table IX

Designation of hem loci of the various haemin auxotrophs

Previous grouping	Genotype	Representative strain	Enzyme defect
I	<i>hemB</i>	1	δ -aminolaevulinate dehydratase
II	<i>hemC</i>	33	PBG-deaminase
III	<i>hemG</i>	321	protoporphyrin-iron-chelatase
V	<i>hemE</i>	64	uroporphyrinogen decarboxylase
VI	<i>hemF</i>	180	coproporphyrinogen oxidase

utilize protoporphyrin IX [1], probably for a similar reason. PBG can neither be used by the proper auxotroph, perhaps because of the metabolite is unable to penetrate into the cell. This is in contrast to the observation of TIEN and WHITE [15] regarding certain auxotrophs of *Staphylococcus aureus* which could be supplemented with PBG.

With the aid of the syntropism test, our haemin auxotrophs could be divided into two groups, the "feeder", which were defective in the first steps of porphyrin biosynthesis and, as a consequence, accumulated ALA, and the "non-feeder" which had a block in a later step of the synthetic pathway.

On the basis of the transductional studies, two groups within the non-feeders could be distinguished. *Hem* loci of the strains belonging to groups I, II and VI proved to be linked to *hemA1*, i.e. these loci could be localized linkage group III of the bacterial chromosome [3], a sector designated by Dubnau *et al.* [16]. On the basis of these results we presume that the genes controlling porphyrin synthesis in *B. subtilis* do not form a single operon and their sequence does not parallel that of the biosynthetic steps. There are some differences in this respect among bacteria which have been studied from this point of view. In the case of *Staphylococcus aureus* [15] the enzyme loci of porphyrin synthesis belong to the same linkage group. It is assumed that the sequence is the same as that of the biosynthetic steps. With *Escherichia coli*, on the other hand, by

mapping the five porphyrin-deficient mutants up to now available [17–19], *hem* loci were shown not to be located in the immediate vicinity of each other on the chromosome. *Hem* loci of each group, as established on the basis of the biochemical studies and the transduction experiments, are paralleled by one enzyme. Preliminary designation of the groups was made with Roman numerals. However, following the suggestions made by DEMEREC *et al.* [20], each gene should be denoted with capital letters of the alphabet. The first locus

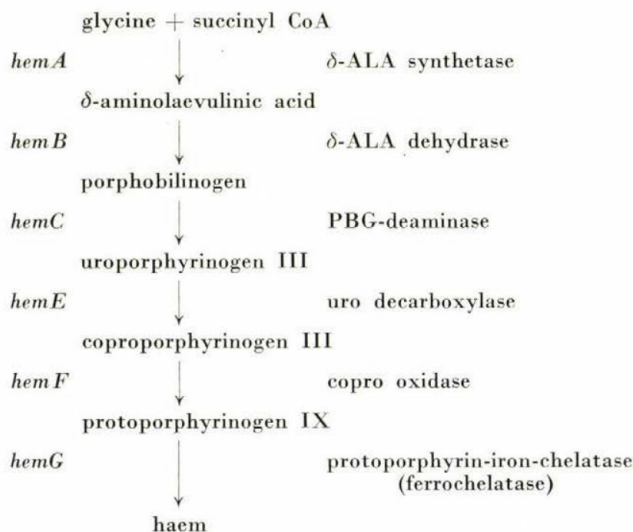


Fig. 1. Identified genes in the porphyrin synthesis of *B. subtilis*. Synthesis of uroporphyrinogen III requires PBG-isomerase in addition to the enzyme given. However, PBG-isomerase is not included in this figure as no mutants defective in this enzyme have been isolated

studied (ALA-synthetase) was designated as *hemA1*, and the present groups too were denoted according to this principle (Table IX).

The genotypes of the strains in group IV could not be determined. It was striking that in the representative of this group (strain IV/344) coproporphyrin III was accumulated to a similar extent as in the parent strain. This fact parallels the observation of GARIBALDI and NEILANDS [21] on the accumulation of coproporphyrin III by wild type *B. subtilis*. It must be emphasized that strain IV/344 could grow only in the presence of haemin.

Fig. 1. represents the identified gene loci and the enzymes corresponding to them. The exact localization on the bacterial chromosome of the gene loci as well as studies on the regulation of porphyrin synthesis are in progress in our laboratory.

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IMMUNE-INDIAN INK METHOD FOR DETECTION OF HEPATITIS A ASSOCIATED ANTIGEN AND ANTIBODY

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Summary. Indian-ink grains coated with commercial gamma globulin (immune-Indian-ink) were agglutinated by 3% of sera from healthy volunteer blood donors; by 4% of those from hospital staff in contact with patients suffering from hepatitis; and by 10% of those from patients with viral diseases other than hepatitis. In contrast, the rate of positive reactions was 86% in the case of sera taken from patients in the acute phase of an illness diagnosed as hepatitis A on the basis of epidemiological and clinical data. Investigation of serum samples taken serially from patients positive in the acute phase of illness revealed that the immune-Indian-ink agglutinating factor does not persist for long in majority of cases. Two months after discharge from the hospital it was present in 18% of the patients only. The reaction proved negative when a limited number of cases diagnosed as hepatitis B were investigated. The immune-Indian-ink agglutinating factor was inhibited by all but one of 36 sera taken in the convalescent phase from patients with a diagnosis of hepatitis A. Some sera displaying agglutination with immune-Indian-ink gave a reaction with uncoated Indian-ink, too. Efforts to free the sera from non-specific agglutinating factor by starch-block electrophoresis have led to partial success. Fractionation on Sephadex G-200 columns suggested that in molecular weight (or particle size) the immune-Indian-ink agglutinating factor is smaller than HB_sAg and larger than the non-specific agglutinating factor. On the basis of these results it is assumed that the immune-Indian-ink reaction is suitable for detecting an antigen tentatively called IH_xAg and its antibody (IH_xAb) specific to hepatitis A.

Since the discovery of the hepatitis B (Australia) antigen (HB_sAg) [1–3], efforts have been made to develop serological methods specific for hepatitis A (infectious hepatitis or IH). DEL PRETE *et al.* [4], using a human serum called Milan I in the double immunodiffusion test, succeeded in demonstrating an antigen in serum samples from patients with acute IH. They stated that this antigen was specific for IH. However, they failed to demonstrate antibody to the antigen in convalescent sera and in subjects with a history of IH [4]. According to ZUCKERMAN [5, 6], the Milan antigen is an abnormal serum lipoprotein which may appear in the blood in the case of hepatic damage, irrespective of its aetiology.

The following facts served as the basis of the present investigations. (i) IH can be prevented or mitigated by administering commercial gamma globulin, which therefore must contain antibody or antibodies to some IH antigen determinants [7]; the same does not seem to be valid for SH; (ii) The immune-Indian-ink reaction (IIR) has been found to be suitable for demonstrating antiviral [8] and antibacterial [9] antibodies. For the detection of anti-HB_s antibody, the method was found to be more sensitive than immunoelectro-osmophoresis [10].

We have assumed that the specific IH antigen (tentatively named IH_xAg), if present in the serum of patients with hepatitis, gives a positive IIR with the corresponding antibody in the commercial gamma globulin preparation. Thus, a micro-agglutination method has been developed using Indian ink coated with antibodies present in commercial gamma globulin. It was tested whether the method would detect an antigen present in the blood during the acute phase of infectious hepatitis, but absent from the blood of healthy subjects or of persons suffering from other diseases; and if so, whether it would be suitable to detect blocking antibodies to IH_xAg present in serum samples of IH patients in the convalescent phase.

During this work in addition to the IH_xAg another agglutinating factor was also found which reacted with non-coated Indian ink. An effort was made to characterize IH_xAg and to distinguish it from the non-specific agglutinating factor.

Materials and methods

Diluent. As diluent veronal buffer of pH 7.3 was used throughout. Solution A contained 4.6 g diethyl-barbituric acid dissolved in 500 ml warm distilled water. Solution B: NaCl, 83.8 g; $NaHCO_3$, 2.52 g; Na-diethyl-barbiturate, 3.0 g; $MgCl_2 \cdot 6H_2O$, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.2 g; dissolved in 1500 ml distilled water. The two solutions were mixed and autoclaved at 121°C for 20 min. The stock solution thus obtained was diluted with 4 volumes of distilled water immediately before use.

Indian ink. Commercial liquid "Holló" Indian ink (Politúr and Vegytermék Co., Budapest) in 3 volumes of diluent.

Gamma globulin. Commercial human 10% gamma globulin, batch No. 810 (Institute for Serobacteriological Production and Research Human, Budapest), diluted 1 : 80. The above dilutions of Indian ink and gamma globulin proved to be the optimum in the case of batches used in the present experiments. The optimum dilution, however, should be determined for each individual batch by pre-titration with known positive sera.

Preparation of immune Indian ink reagent (IR). Equal volumes of the working dilutions of gamma globulin and Indian ink were mixed in a small round-bottomed flask using a "Vibrotherm" shaker (Labor MIM, Budapest) at 120 rpm at room temperature for 30 min. Then an equal volume of diluent was added. The reagent thus prepared could be stored at +4°C for several weeks.

Immune Indian ink reaction (IIR) for titration of IH_xAg . All titration procedures were carried out in the V-shaped wells of TAKÁTSY's Microtiter trays [11]. Two drops (0.05 ml) of diluent were placed in the first well of each row and one drop (0.025 ml) in each of the others. Six dilution series (1 : 3–1 : 96) were prepared from native sera or from separated serum fractions using the 0.025 ml dilutor. One drop of IR was added to each serum dilution. After 30 min gentle shaking at room temperature and 90 min incubation at 37°C in a wet chamber without shaking, the results were read with the naked eye; a well-defined partial agglutination was regarded as the limit dilution. A prozone phenomenon was often observed.

IIR for IH_xAb titration. Human serum previously found to contain IH_xAg was used as antigen. Four to 8 agglutination units of antigen were added to halving dilutions of the serum under investigation. In choosing the proper antigen dilution, the prozone phenomenon observed in antigen titration was taken into consideration and an antigen control was examined simultaneously with each experiment. The trays were gently shaken for 20 min, then IR was added as indicator of the free IH_xAg . The further procedure was the same as described above. As IH_xAb titre, the final serum dilution was taken in which at least a partial agglutination could be seen.

Demonstration of IIR. Fig. 1 shows a Microtiter tray. In rows 1–6, six positive sera had been titrated for IH_xAg . The positive agglutination pattern is clearly distinguishable from the negative one. The titres are between 1 : 24 and 1 : 96. A prozone phenomenon is seen in each row. In rows 7–12, six convalescent sera were titrated for IH_xAb using as antigen

the same patient's IH_xAg positive acute-phase serum. Blocked reactions indicating the presence of anti- IH_x are well distinguishable from the positive agglutination. The blocking titres were variable.

Non-specific Indian ink aggregation. As a control, the same procedure as described under "Titration of IH_xAg " was carried out with uncoated Indian ink.

Column chromatography. Columns 3.5×50 cm in size with Sephadex G-200, equilibrated with diluent supplemented with 0.01% methiolate, were employed. Fractions 4–8 ml in volume, depending on the properties of the preparation, were collected by an automatic apparatus. Protein content was estimated by measuring optical density at 280 nm in a PYE Unicam SP-1800 spectrophotometer.

Agarose electrophoresis. Agarose (0.9%) was dissolved in pH 7.6 PRINCE-buffer [12]. The solution was layered in 2 mm thickness on a 6×9 cm glass plate. A narrow trough was

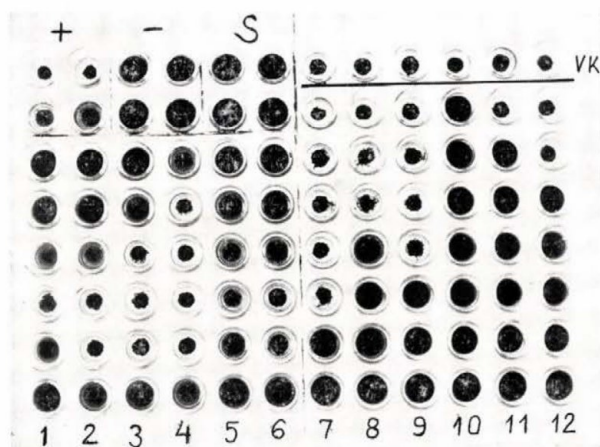


Fig. 1. Immune Indian ink reaction and blocking reaction in a Microtiter tray. 1–6: Immune Indian ink agglutination; 7–12: blocking reaction. In the upper left corner + indicates the 1:3 and 1:6 dilutions of a control serum, which had proved to be positive for IH_xAg ; sign — indicates the same dilutions of a previously antigen-negative serum; "S" indicates the immune Indian ink system; "VK" indicates the antigen control in the six blocking reactions

made across the longitudinal axis and 0.3 ml of the sample to be tested was placed in the trough. This system was run at 100 V/26 mA at room temperature for 4 hr. Then slices 1 cm in thickness were extracted in 3 ml diluent each. The location of protein in the gel was demonstrated by staining a longitudinal strip with 1% Amido black 10B, using acetic acid differentiation.

Starch-block electrophoresis. A block was prepared from carefully washed starch suspended in PRINCE-buffer in a container $8.5 \times 13.5 \times 0.8$ cm in size. A trough 1 cm in width was made parallel to the longitudinal axis. In the trough 4 ml serum and several drops of 1% bromophenol-blue were placed. Then the trough was filled up to the level of the block with starch suspension. The serum was run at 120 V/40 mA at a temperature of $+4^\circ C$ for 5 hr and 30 min. Slices 1 cm in thickness were cut out of the block and extracted with 2.5 ml diluent. From the extract the starch was sedimented by centrifuging at 3000 rpm for 10 min and the supernatant was tested for antigen.

HBAg was titrated by the complement fixation (CF) test as described earlier [13, 14]. All the sera were examined by this method. The double-blind system was applied throughout.

Donors of sera. 1. *Children with acute IH.* 170 inpatients in a Budapest hospital, from 1 to 14 years of age; of these, 120 had been involved in IH outbreaks. In the remaining 50 cases the diagnosis of IH was based on clinical signs. Samples were taken serially in 10–12 day intervals from the day of admission till discharge. An additional sample was taken 2 months later.

2. *Control hospitalized children.* From 143 inpatients in the same hospital, suffering from measles, rubella, chicken pox or other acute viral disease, one blood sample each was collected at admission.

3. *Hospital staff.* One serum sample was collected from 315 healthy employees each of a hospital in Miskolc. This town and its surroundings were afflicted by an IH outbreak at the time of blood sampling. In the hospital, many patients with SH infection were treated.

4. *Healthy blood donors.* Six hundred and fifty-three blood samples from unselected volunteer donors screened in Budapest in December, 1972, and 24 blood samples from HB_sAg positive donors selected from those screened out in November, 1972.

Results

Occurrence of IH_xAg and HB_sAg in blood samples from control persons. Results of the tests carried out with blood samples from healthy blood donors, hospital staff and non-hepatitis patients are shown in Table I.

Among the 653 non-selected donors, six proved to be HB_sAg positive and 20 IH_xAg positive. None of them was double-positive. The clinical laboratory tests were negative for all the IH_xAg positive donors except one, who had an elevated SGOT level. This donor was hospitalized with IH one week after screening. Of the 24 selected HB_sAg positive donors, two were carrying IH_xAg as well.

Of the 315 serum samples collected from hospital staff in Miskolc, 14 contained IH_xAg and 30 contained HB_sAg. One sample contained both. All the members of the staff were in contact with patients many of whom were suffering from IH or SH.

Among the children suffering from acute viral disease other than hepatitis 15 (10.5%) had IH_xAg and two (1.4%) had HB_sAg in their serum.

Table I

Occurrence of IH_xAg and HB_sAg in healthy subjects and patients with viral disease other than hepatitis

Source of blood sample	No. of tested persons	IH _s Ag		HB _s Ag		HB _s Ag and IH _s Ag simultaneously		
		positive				positive		negative
		No.	per cent	No.	per cent	No.	per cent	No.
Volunteer blood donors, Budapest								
Unselected donors, December, 1972	653	20	3.1	6	0.92	0	.	627
HB _s Ag positive donors screened out in November, 1972	24	0	.	22	.	2	.	0
Hospital staff, Miskolc	315	14	4.4	30	9.5	1	0.31	270
Children with non-hepatitis viral disease, Budapest	143	15	10.5	2	1.4	0	.	126

Table II

Occurrence of IH_xAg and HB_sAg in serial serum samples from children with acute IH

Results	Serial number of blood									
	I		II		III		IV		V	
	No.	per cent	No.	per cent	No.	per cent	No.	per cent	No.	per cent
IH_xAg positive	131	77	115	80	71	64	17	48	18	18
IH_xAg and HB_sAg positive	16	10	3	3	3	3	0	0	0	0
Negative	23	13	25	17	36	33	18	52	83	82
Total	170	100	143	100	110	100	35	100	101	100

Samples I-IV were taken at 10-12 day-intervals during acute illness

Sample V was taken 2 months after discharge

Occurrence of IH_xAg and HB_sAg in serum samples of children with IH.

The 170 children with a clinical diagnosis of IH included 136 girls and 34 boys (see Table II). A hundred and twenty had been involved in outbreaks of IH, in 50 cases the anamnestic data were incomplete. In the former group, 106 (88%), in the latter, 41 (82%), displayed IH_x antigen in one or more serum samples.

The number of positive samples was the highest at the first sampling *i.e.*, on the first or second day after admission. It was less at the second sampling and showed a considerable decrease thereafter. Two months after discharge 18% of the 101 re-examined children were positive.

The IH_xAg titres varied between 1:6 and 1:96, showing no close relation to the severity of the illness. On the other hand, an increasing tendency of, or no change in the IH_xAg titre during the disease usually indicated an unfavourable clinical course. There was no relation between IH_xAg titre and any of the usual laboratory tests, except a loose one with the thymol turbidity test.

It is of interest how the positive reactions for IH_xAg and HB_sAg occurred simultaneously in the course of the illness. All the 16 children positive for both IH_xAg and HB_sAg at admission remained positive for IH_xAg had become negative for HB_sAg by the second sampling. Three patients positive for both antigens at the second sampling had been IH_xAg positive and HB_sAg negative at admission. Of these, only one was still double-positive at the third sampling, by which time two previously IH_xAg positive and HB_sAg negative children became IH_xAg positive and HB_sAg positive.

During the period of the study, seven children were admitted to the same hospital with a diagnosis of SH. All of them proved HB_sAg positive, but IH_xAg negative.

Tests for anti-IH_xAg. There were 56 IH_xAg positive patients whose serum soon became free of IH_xAg. Thirty six convalescent samples of these patients were tested for anti-IH_x using as antigen the IH_xAg positive acute-phase serum of the same patient. All but one of the convalescent sera proved positive in this blocking test. Each IH_xAb positive serum had blocking activity when it was tested against IH_xAg containing serum taken from another person. There were, however, some titre differences, in that the titres were somewhat higher when the antigen and antibody had originated from the same person.

Non-specific Indian ink aggregation. As a control, 361 sera including 86 IH_xAg negative ones and 275 IH_xAg positive ones, were tested with uncoated and gamma globulin coated Indian ink suspensions in parallel. The uncoated suspension was agglutinated by none of the IH_xAg negative sera, among them 28 were displaying high-titre serum lability tests. Of the 275 IH_xAg positive sera, 60 failed to aggregate the uncoated Indian ink suspension, 103 aggregated it up to a dilution lower than the corresponding titre with the gamma globulin coated suspension. In 112 of the IH_xAg positive cases (41%), non-specific agglutination was of the same titre as (89 sera), or higher by one dilution step than (23 cases), the IH_xAg reaction. The quotient of the two titres often varied in the samples taken from the same patient at different times.

The non-specific Indian ink aggregation, too, was inhibited by convalescent sera. This reaction was carried out with 34 convalescent sera. The titre was identical with the anti-IH_xAg titre in 18 cases. It was lower in 8 cases and higher in 8 cases.

Efforts to characterize the agglutinating factors. Column chromatography. A typical experiment is shown in Fig. 2. The non-specific Indian ink aggregation titre of the serum was 1 : 96, as high as its IH_xAg titre.

The first IH_xAg fractions were recovered from the Sephadex G-200 column together with the first protein fractions. In the first seven fractions the quotient IH_xAg/non-specific aggregating factor approximated 4. For the following 10 fractions this quotient was approximately 1.

Fig. 3 shows a similar experiment with the mixture of a relatively low-titre IH_xAg positive serum and an HB_sAg positive serum. The bulk of the HB_sAg was recovered before and together with the first protein peak but, probably due to a modifying effect of the IH_xAg, the fractions between the first and the second protein peaks were not entirely free from HB_sAg. The first three IH_xAg positive fractions had only specific titres, five subsequent fractions had the same specific and non-specific titres.

Agarose-gel electrophoresis. A mixture of equal volumes of a HB_sAg

positive serum with a CF titre of 1:60, and of an IH_xAg positive serum with an IIR titre 1:24 was run. The HB_sAg , as expected, moved towards the positive pole whereas IH_xAg was recovered from the fraction corresponding to the trough or was slightly shifted towards the negative pole. The titres did not exceed 1:2, i.e., the degree of elution was low.

Starch block electrophoresis. The proteins staining with bromophenol-blue migrated towards the positive pole and nearly the whole non-specific Indian-ink-aggregating activity was found in these fractions. The specific activity, on the other hand, was nearly quantitatively recovered from the

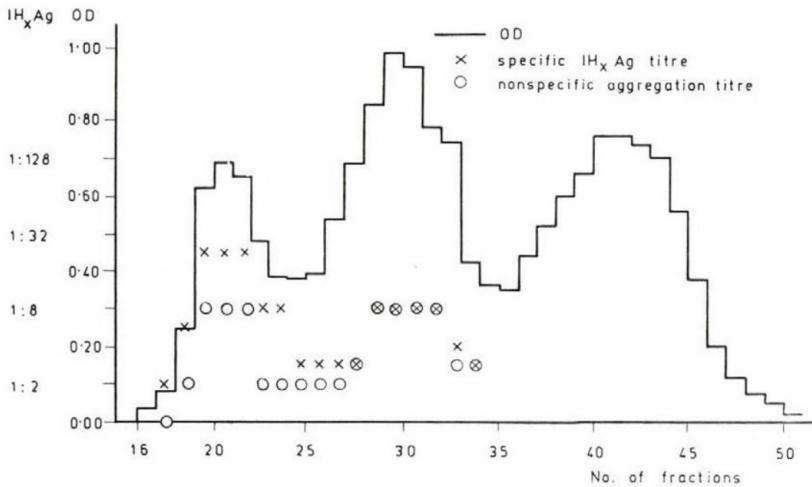


Fig. 2. IH_xAg positive serum fractionated on Sephadex G-200 column

trough and the gel between the trough and the negative pole. These latter fractions contained only traces of non-specific activity.

Resistance of IH_xAg . IH_xAg lost the bulk of its activity on lyophilization of the full serum. In the fractions recovered from the Sephadex G-200 column or by electrophoresis, the IH_xAg resisted lyophilization. There was a great individual variation in its resistance to freezing and thawing.

Three hundred and fifty-four IH_xAg positive serum samples were stored at $-40^{\circ}C$ for three months. Of these, 29% lost their titre entirely, 41% partially, whereas in 30% of the samples the IH_xAg titre remained unchanged, or even slightly increased. The inactivation may have been due to the joint effect of freezing, storage and thawing.

Ten sera were stored at $+4^{\circ}C$ and subjected to titration at monthly intervals. These sera retained their original titres for at least three months. In another experiment at $+4^{\circ}C$, the sera lost the bulk of their IH_xAg titre within three months.

Twenty-five serum samples were heated at 56°C for 30 min. Of these, 18 had entirely, and three partially lost their IH_xAg activity, in four cases the titre remained unchanged. The last seven sera had relatively high non-specific aggregation titres.

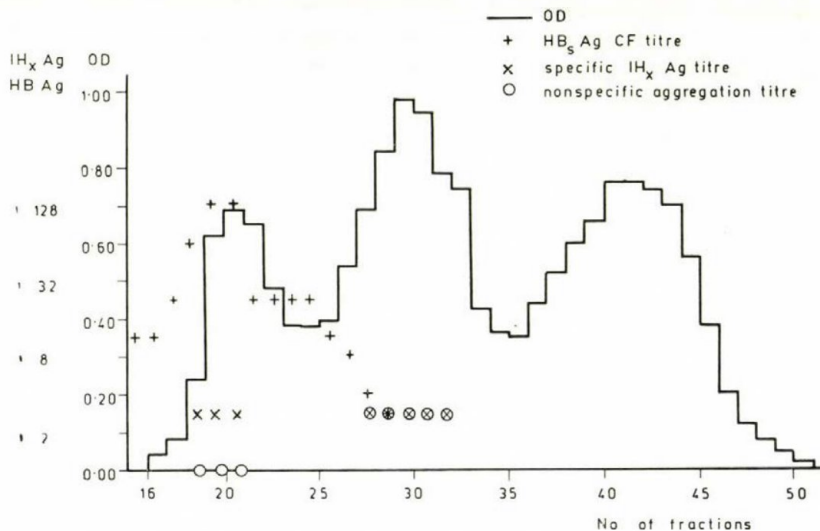


Fig. 3. Mixture of HB_sAg positive serum and IH_xAg positive serum fractionated on Sephadex G-200 column

Discussion

Commercial human gamma globulin preparations contain a great variety of antibodies, among them those which can prevent or mitigate IH. We coated Indian ink grains with such a preparation and developed a micro-agglutination method to study IH associated antigen(s).

The reaction was positive with more than 80% of the first serum samples from children in the acute phase of IH. The factor or factors responsible for this reaction, tentatively called IH_xAg , disappeared during convalescence in the majority of cases and the convalescent sera contained an anti- IH_xAb capable of blocking the agglutination of the gamma globulin coated Indian ink grains.

In contrast, the gamma globulin coated Indian ink grains were agglutinable with only 3–4% of the serum samples obtained from healthy adults and 10% of those from children suffering from acute viral disease other than hepatitis. Even the sera from children with SH gave negative results.

Thirteen per cent of the acute-phase sera obtained from children with IH failed to agglutinate the coated grains. These cases of hepatitis may have

been caused by some other viruses or the quantity of the circulating antigen had fallen below the sensitivity of our test.

The rate of IH patients giving a positive IIR was high in the early phase, but decreased markedly in the course of the illness. Furthermore, during convalescence an antibody could be detected against the antigen present in the acute phase. These data show that factors specific to IH are involved in the reaction.

Many sera giving a positive IIR also agglutinated non-coated Indian ink. The factor(s) responsible for this aspecific reaction proved, however, different from IH_xAg on the basis of column chromatography and of starch electrophoresis.

A question to be elucidated is the blockade of the non-specific Indian ink aggregation by convalescent sera.

It is certain that the gamma globulin coated Indian ink grains are agglutinable with a great variety of antigens. The number of antigens coming into account is, however, highly reduced if human serum is used as the source of antigen. The role of the Rheumatoid factor and of LP-X antigen in aggregation of coated and uncoated Indian ink is under investigation.

Besides the IH_xAg , HB_sAg was also present sometimes either in the acute phase or during convalescence, and disappeared before the next sampling. Technical errors could be ruled out since in CF tests for the detection of HB_sAg the same serum was used throughout. It is a possibility, however, that our serum contained antibodies not only to HB_sAg but also against antigens released owing to impaired liver function during the disease. In faecal samples from IH patients, however, immunologically IH-specific particles and particles with double-specificity have been detected [15, 16]. It cannot be excluded that the latter particles appear in the blood transiently in a certain phase of the illness. Double infection might be another explanation, however unlikely at such a high frequency.

There is no doubt that our antigen as well as the previously described ones [4, 15, 16] showed a fair association with the epidemiological-clinical diagnosis of type A hepatitis. It seems to be an advantage of our method that it permits quantitative studies and is suitable for differentiating the presumably specific antigen from non-specific ones.

Further investigations are needed for clarifying the exact nature and origin of the antigen found by us in IIR.

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NOTE

SEROVARIANTS OF *LISTERIA MONOCYTOGENES* AND OTHER *LISTERIA* SPECIES*

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The original antigenic scheme of *Listeria monocytogenes* has been supplemented by the recognition of further antigen combinations mainly within the original serovar 4 (PATERSON) of this species as well as the finding of additional serovars and of a new biovar strongly related to *Listeria grayi* by WELSHIMER and MEREDITH [1]. The latter authors have raised this biovar to the rank of a species (still *sub judice*) and named it *Listeria murrayi*. It is mainly differentiated from *L. grayi* by its inability to reduce nitrate to nitrite. Both species share the ability to break down mannitol. Serologically, the two species appear strongly related if not identical, but they are different from all *L. monocytogenes* serovars.

Therefore the antigenic scheme published by SEELIGER [2] needs adjustment to the present state of knowledge. Moreover this scheme has led to some misunderstanding relating to the serological position of serovar 4ab which was listed under PATERSON's original serovar 3.

This is corrected in Table I which lists serovar 4ab of *L. monocytogenes* properly under serovar 4 of PATERSON and includes the antigenic formulas of serovar 4g.

The following antigen combinations have been identified by agglutination tests, but have not yet been listed as they still need clarification and absorption studies (see Table II).

For the sake of completeness the antigenic formulas of *L. grayi* and *L. murrayi* (*sub judice*) are included.

* Supplement to a table published in Acta microbiol. Acad. Sci. hung. 19, 274 (1972).

Table I

Serovars of L. monocytogenes and their relations to L. grayi and L. murrayi

Designation		O antigens										H antigens
PATERSON	SEELIGER-DONKER-VOET											
1	1/2a	I	II	(III)								AB
	1/2b	I	II	(III)								ABC
2	1/2c	I	II	(III)								B D
3	3 a		II	(III)	IV							AB
	3 b		II	(III)	IV							ABC
	3 c		II	(III)	IV							B D
4	4 a			(III)	(V)		VII		IX			ABC
	4 ab			(III)	V	VI	VII		IX			ABC
	4 b			(III)	V	VI						
	4 c			(III)	V		VII					ABC
	4 d			(III)	(V)	VI		VIII				ABC
	4 e			(III)	V	VI		(VIII)	(IX)			ABC
	4 f			(III)	V						XV	ABC
	4 g			(III)	V	VI	VII		X	XI		ABC
	5			(III)	(V)	VI		VIII		X		ABC
	6			(III)			VII	VIII			XI	ABC
	7			(III)							(XII) XIII	ABC
	<i>L. grayi</i>			(III)							XII XIV	E
	<i>L. murrayi</i>			(III)							XII XIV	E

Table II

Further antigen combinations of L. monocytogenes as observed in Würzburg; provisional further serovars of O groups 4 and 6

No. of tested strains	O antigens										H antigens
1	(III)	V	VI	VII							ABC
1	(III)	V	VI	VII	VIII						ABC
1	(III)	V	(VI)	VII			XI	XII			ABC
1	(III)	V	VI		VIII	X	XI				ABC
6	(III)	V	VI			X	XI				ABC
2	(III)	V		VII						XV	ABC
3	(III)	V			VIII						ABC
3	(III)	V			VIII	X	XI				ABC
2	(III)	V				X	XI				ABC
1	(III)		VI	VII		X					ABC
1	(III)			VII		X	XI				ABC

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SIXTH CONGRESS OF THE HUNGARIAN SOCIETY OF MICROBIOLOGY

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ABSTRACTS OF PAPERS

Genetics

PRESENT STATUS OF MEGACIN RESEARCH: LYSOGENIC CONVERSION IN *BACILLUS CEREUS* INDUCING PHOSPHOLIPASE A PRODUCTION

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Megacin, an agent discovered and characterized by IVÁNOVICS and his collaborators, was identified with phospholipase A by Japanese authors. More recently, several *Bacillus cereus* strains have been found which are lysed in the presence of mitomycin; the lysates thus obtained contained a bacteriocin, termed megacin A; this was biologically closely similar to megacin. A detailed study of one of the strains, *B. cereus* W, produced megacin A being also identical with phospholipase. *B. cereus* W carries two prophages, the loss of one of which is accompanied by the loss of the enzyme. The lysate of the wild-type bacterium proved to be plaque-forming in the cured derivatives (*cin*⁻ cells). The plaque-forming phage (wx) has proved extremely specific to the cured derivatives: the authors failed to propagate it in a great number of strains of various species of the genus *Bacillus*. The *cin*⁻ cells are lysogenized by the wx phage and the lysogenized cells produce phospholipase A.

GENETICS OF COLICINOGENIC FACTORS

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Colicinogenic (*col*) factors are genetic units governing synthesis of highly specific antibiotic proteins (colicins) in *Enterobacteriaceae*. They can be transferred from one strain to another by conjugation as well as by transduction. They generally appear as extrachromosomal determinants but integration into the chromosome may occur in some cases. They are clearly related to other plasmids such as the F agent and the R factors of transmissible antibiotic resistance. *Col* factors are essentially composed of structural genes for the synthesis of one or several proteins (colicins), of genes governing immunity to the colicin(s) produced, of regulator genes and of genes necessary for autonomous replication. Such simple *col* factors appear as covalently closed circular DNA molecules averaging 5×10^6 in molecular weight. They are therefore

similar to the non-self-transferable segregants derived from more complex R factors. Most R factors are however self-transferable but several *col* factors are also endowed with fertility. They include a transfer segment which appears to be identical with that of an R factor and extends to about 5×10^7 molecular weight. In both cases this segment is carrying genes responsible for the ability to confer maleness, conjugal self-transferability as well as an ability of transferring other plasmids or chromosomal segments, interference with other plasmids (superinfection immunity and mutual exclusion), and eventually a restriction of certain bacteriophages and an increased resistance to UV irradiation. The relationship between *col* and R factors is further supported by the fact that they can recombine together and that R factors carrying colicin genes are not uncommon in nature.

RECENT FINDINGS ON THE GENERAL MODE OF ACTION OF COLICINS

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In 1964, NOMURA formulated the general theory of the mode of action of colicins on sensitive bacteria. This theory seems to be open to revision in the light of recent findings. It has been proved that L-forms of the protoplast type may display a lower, an identical or even a higher degree of sensitivity to colicins than rods of the same strains. In certain cases the loss of the cell wall establishes sensitivity in a previously resistant strain. Isolated membrane vesicles from colicin-resistant strains become sensitive to colicin, while vesicles from colicin-tolerant strains are unaffected by it. Large amounts of colicins may be bound to structures other than effective receptors on the surface of sensitive cells. Considerable defects in cytoplasmic membranes have been detected in colicin-tolerant mutants. The specific biochemical effects of two colicins — E2 and E3 — have been proved not only *in vivo*, but also in isolated cell fractions *in vitro*; *in vitro*, colicins are active not only on fractions of sensitive cells, but also on those of resistant, tolerant and even heterologous ones. Thus, two kinds of colicin receptors should be distinguished: (i) specific structures on the cell wall, able to bind certain colicins, and (ii) specific sites on the cytoplasmic membrane, the contact of which with colicin molecules is biologically effective; the latter are more specific than the former. Susceptible cells must possess both kinds of receptors: in immune cells the membrane receptors may be “switched-off” functionally; cells without wall receptors (and in many cases also without membrane ones) would be resistant and cells without membrane receptors would be tolerant.

MODE OF ACTION OF ENTEROCIN A

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The effect of enterocin A, a bacteriocin produced by *Streptococcus faecium* El, on the synthesis of DNA, RNA and protein, the active transport of isoleucine, and the ultrastructural alterations in *S. faecium* 158 were studied. The bacteriocin strongly inhibited the incorporation of labelled isoleucine, thymidine and uracil into protein, DNA and RNA, respectively, as well as the accumulation of labelled isoleucine and caused rapid exit of previously accumulated isoleucine. These results indicate that enterocin A inhibits macromolecular synthesis and blocks certain permease activities. Treatment with enterocin A caused extensive morphological changes in the ultrastructure of *S. faecium* 158. Nucleoid DNA normally seen as fine filaments scattered throughout the cell, was aggregated into dense DNA masses. These changes were followed by a dissolution of the cell content resulting in bacterial ghosts composed of cell walls with remnants of the cytoplasmic membrane and internal structures.

REPLICATION OF STRINGENTLY AND RELAXEDLY REGULATED
R PLASMIDS

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The conjugative R factors, R28K (44 megadaltons) and R6K (26 megadaltons) each carry resistance to penicillin, but differ in the control of their replication; R28K is defined as a plasmid stringently controlled in replication to about one plasmid molecule per chromosome, whereas R6K is defined as relaxed, being present in approximately 12 copies per chromosome, and increasing to about 40 copies in stationary phase cultures (KONTOMICHALOU *et al.*, 1970). These two factors also differ in their replication behaviour under other conditions. Following a medium "shift-up", there is a rapid fourfold increase in the numbers of copies of R6K decreasing to the normal level after two host cell generations, whereas R28K increases only twofold and declines to the normal level after one generation. Following the addition of chloramphenicol (150 $\mu\text{g/ml}$), replication of both factors decreases parallel with that of the chromosome. Inhibition of R factor and chromosomal replication was found at non-permissive temperatures in host strains carrying mutations in the *dnaB*, *C* or *D* loci. In *dnaA* or *dnaE* mutants, R28K replication was also rapidly inhibited at the non-permissive temperature, whereas replication of R6K

continued, reaching after five hours a fivefold increase in *dnaA* mutants and an eighteenfold increase in *dnaE* mutants. Control experiments using F and *colE1* factors showed an inhibition of the F factor replication similar to that of R28K under all the above conditions. *ColE1* was superficially similar to R6K, replicating both in *dnaA* and *dnaE* mutants, although its replication was less extensive, reaching only a three to fourfold increase in both mutant bacterial hosts. Thus, the replication of stringent factors appears to be controlled in a closely coordinated way with that of the chromosome, all mutations affecting chromosomal replication similarly affecting the replication of the stringent factors. In contrast, relaxed factors such as R6K, do not appear to require DNA polymerase III activity (absent in *dnaE* mutants) nor the continued presence of the initiator factor produced by the *dnaA* gene. Other results have shown more extensive replication of the R6K factor under conditions selecting for penicillin resistance. After 60 generations of growth in 1 µg/ml penicillin, the number of copies of R6K increased to approximately 70-fold then in the next 40 generations in the absence of penicillin it was reduced to the "standard" thirteenfold number. Under similar conditions there was no marked increase in the number of copies of the R28K plasmid, which were maintained in an approximate 1 : 1 relationship with the chromosome.

CONJUGAL TRANSFER OF *colE1*⁺ AND *colE1*⁻ CHARACTER

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A *colE1*⁺ streptomycin sensitive (*str*^S) donor strain of *E. coli* was crossed with a threonine, leucine, arginine and histidine multiauxotrophic *str*^r *colE1*⁻ recipient. The frequency of the conjugal transfer of *colE1*⁺ character, as a non-selective marker, was investigated in the *str*^r *threo-leu*⁺ or *str*^r *arg*⁺ recombinants. It was found that 32% of the arginine independent and 18% of the *threo-leu* independent colonies were *colE1*⁺. In the reverse cross, i.e., *colE1*⁻ donor and *colE1*⁺ recipient, *colE1*⁻ recombinants have been obtained. Although it is generally accepted that the *colE1* factor exists in *E. coli* only as an extrachromosomal element (plasmid), the results cannot be explained in this way. It is suggested that *colE1* factor may integrate into the chromosome of *E. coli* in addition to its well-characterized plasmid occurrence.

COMPARATIVE STUDY OF EI AND V COLICIN FACTORS
IN RECOMBINATION SYSTEM

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Two colicin factors, the F-like *colV2* (from *E. coli* K-94) and *colEI-ML*, were studied in *Hfr* $\times$ *F*⁻ crosses and the recombinants were selected for threonine-leucine and arginine markers on M9 minimal media with streptomycin. In the case of colicin V, transfer to the recombinants was nearly 100%. In the *Hfr* Cavalli/V $\times$ PA309 (λ) V⁺ crosses, *Hfr* became partly V colicinogenic. The presence of *colV* did not disturb the chromosomal transfer. *ColEI* transfer took place in about 40–50%, although where the recipient strain was colicinogenic, the *arg*⁺ recombinants were drastically reduced. In such conjugations where both strains were *col*⁺, the same data were obtained as those found in the above mentioned cases. On the basis of the results of experiments with *Hfr* CV⁺ $\times$ PA309/ λ /EI⁺, *colEI* was stably transmitted into the daughter cells, besides they gained V⁺ colicinogenic properties in a normal way. These results did not verify the effect of *colV* on *colEI* which is supposed to be of a chromosomal integrated nature. Studying the cotransfer of the *col* factors EI + V from male to noncolicinogenic female showed differences between the two markers, nevertheless the *arg*⁺ recombinants acquired much more double colicinogenic properties. The situation was completely different in reciprocal crosses where the *F*⁻ strains were double colicinogenic and the donor strains noncolicinogenic. Among these recombinants EI-V⁺ colonies occurred frequently, especially if *arg* was the selected marker.

TRANSFORMATION OF STAPHYLOCOCCI

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The basic difficulties of staphylococcal transformation are: (i) the difficulty of destroying the cell wall, due to its resistance against the action of detergents and enzymes, which makes it difficult to obtain DNA-preparations of high molecular weight; (ii) the selection of suitable nutrient media and the conditions of cultivation of the recipient cells, optimal for the production of a competent population. A new method has been developed for the isolation of staphylococcal DNA. The DNA-preparations are characterized by their biological activity, molecular weight, protein content and absorption spectrum.

The optimum conditions for obtaining competent cells have been estimated. It was found that the competence of staphylococci appears in the 4th hr of cultivation and disappears in the 6th hr. The maximum of competence coincides with the end of the exponential growth phase. Experiments on urographin density gradient showed that the competent cells were recovered in the light fraction and that a transformation rate of 4.3×10^{-4} could be achieved. Competent cells were found to differ from incompetent ones in some properties such as isoelectric point, resistance to antibacterial agents, growth curves, etc. Assuming that the low staphylococcal transformation rate is due to the peculiar surface structure, it was attempted to increase cell permeability by treating the cells with enzymes and surface-active agents. Of the agents used, trypsin had a positive effect in that the transformation rate increased up to 10^{-3} . The possible explanation of this phenomenon is discussed.

STUDIES ON THE CONVERTING PHAGES OF SHIGELLA FLEXNERI

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This group of bacteriophages is responsible for the appearance of α -glucosyl chains which represent the type-specific antigen determinants of *Shigella flexneri*. These phages constitute a homologous group in respect of their uniform electron microscopic and plaque morphology, the lack of UV inducibility, host range, and cross neutralization by antiphage sera. Furthermore, this phage group forms a homo-immune system. Studying the phenotypic expression of the converted I, IV₁, V and 7,8 antigens in dilysogenic derivatives, an inhibition was observed between some of these antigens, which could not be related either to an antigen masking effect, or to phage exclusion. On the basis of the observed immune repressor effect, the converting prophage status seems to be a special case of lysogeny.

COMMON GENETIC DETERMINANCE OF NITROGENASE AND NITRATE REDUCTASE IN RHIZOBIUM MELILOTI

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Synthesis of the nitrogenase enzyme system in bacteroids is the most important regulatory step in symbiotic nitrogen fixation by *Rhizobium*. To study the genes determining this process, the relations were investigated

between the nitrate reductase enzyme system and the N_2 -reducing nitrogenase. Sixteen nitrate reductase-free *R. meliloti* mutants were isolated after treatment with N-methyl-N'-nitro-N-nitrosoguanidine. The change in enzyme function of these mutants was compared to that of the wild type. Plants were infected with these mutants and the nitrogenase activity was measured. In six mutants the lack of nitrate reductase activity went parallel with the lack of nitrogenase activity. An *in vitro* system was worked out for the molecular identification of the enzyme components and for functional studies of the common electron transport steps. According to the correlation established between the nitrate reductase and nitrogenase enzyme systems, isolation of nitrate reductase mutants proved to be a suitable method for the selection of nitrogenase-free bacteria.

DEVELOPMENT AND TRANSFERABILITY OF ANTIBIOTIC RESISTANCE IN ESCHERICHIA COLI AND SOME SALMONELLA SPECIES

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Adaptive oxytetracycline (OTC) resistance developed in *Escherichia coli* and some *Salmonella* and *Staphylococcus* species in several weeks within 17–59 subcultures. OTC resistance developed gradually, its level varying between 25 and 200 $\mu\text{g/ml}$. Parallel to the development of OTC resistance, chloramphenicol cross-resistance of a 25–50 $\mu\text{g/ml}$ level also developed in one *E. coli* and in one *S. typhi-murium* strain. When the bacterial strains were subcultured in antibiotic-free media, OTC resistance disappeared by the 17th passage. Of 61 *E. coli* strains isolated from suckling piglets and calves died of *E. coli* dyspepsia, 27 were resistant to OTC alone while 25 showed — though in varying distribution — resistance to 2–5 antibiotics, viz. streptomycin, OTC, neomycin, chloramphenicol, and furadantin. The resistance of the *E. coli* strains — both single and multiple — could be transferred to recipient *E. coli* and to recipient *S. typhi-murium* at a rate of 30 and 5–6%, respectively. The level of the resistance transferred was above 200 $\mu\text{g/ml}$ and persisted through 20 *in vitro* passages.

CHARACTERIZATION ON THE BASIS OF PHAGE RESTRICTION
OF R FACTORS DERIVED FROM ESCHERICHIA COLI STRAINS

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Forty-five per cent of the concomitant *Escherichia coli* strains isolated from patients suffering from conditions caused by pathogenic or presumably pathogenic *E. coli* and shigellae, and of *E. coli* strains isolated from foodstuffs proved resistant to two or more antibiotics. R factor was demonstrated in 78% of the multiple-resistant strains. Crosses were performed mostly in parallel, using two recipient strains, viz., *E. coli* HfrH *nal^r lac⁻* and *E. coli* K12 W1 — A2 (*lac⁻*) *F⁻*. Of the 39 R factors, 31 were *fi⁻* and 8 were *fi⁺*. Classification of the R factors has been successful with phages T and P₁ and 28 *E. coli* type phages on the basis of their restriction properties. Similarly to the incompatibility grouping of the *fi⁻* R factors proposed by DATTA and HEDGES, the grouping based on the phage restriction pattern is suitable for the classification of the R factors occurring in *E. coli* strains and can be applied in studied of the spreading of R factors.

R FACTORS DERIVED FROM SHIGELLA FLEXNERI STRAINS

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Shigella flexneri strains isolated from patients in Hungary in 1972 were tested for resistance to antibiotics. Out of 845 strains, 227 (27%) were resistant to 1–3 antibiotics. Resistance was due to R factors in 79%. In contrast to the data of YOSHIKAWA, only 11% of the examined R factors proved to be *fi⁺*. The R factors derived from *S. flexneri* strains were transferred into a sensitive *E. coli* and a *S. flexneri* strain and phage restriction was examined. An *E. coli* phage set containing 28 phages (MILCH) and a *S. flexneri* phage set containing 19 phages (LÁSZLÓ–MILCH) were used. On the basis of phage restriction (using the *S. flexneri* phage set) and relative efficiency of plating, R factors were classified into 9 types. Analyzing the phage-type distribution of *S. flexneri* strains, in 1972 R factors with broad restriction pattern were found to be carried by all of the examined strains of phage types 74, 76 and 55. These phage types had probably developed as a result of R factor inclusion. A number of strains carrying R factors of broad restrictive ability were sensitive to phages, restricted by the same R factor when it was carried by an other recipient strain. The expression of phage restriction of the examined R factors depended on the recipient strain.

ANTIBIOTIC RESISTANCE FACTORS OF SALMONELLA TYPHI-MURIUM STRAINS ISOLATED FROM VARIOUS SOURCES IN HUNGARY IN 1971-1973

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Resistance to one or more antibiotics was found in 90 of the 2059 strains studied. Of the 90 resistant strains 60, 23 and 7 were of human, animal, and water origin, respectively. The most frequent resistance markers were tetracycline, streptomycin and tetracycline-streptomycin (27, 23 and 11 strains, respectively). In 40 of the 46 strains studied, antibiotic resistance could be transferred by conjugation to the isogenic HfrH, K-12 and Row strains of *Escherichia coli* and then to the LT-2 strain and to the standard strain of phage type 1 (FELIX-CALLOW's series of phages) of *S. typhi-murium*. In the above strains, phage restriction was studied by the phage series developed by MILCH and introduced by FELIX and CALLOW for the phage-typing of *S. typhi-murium*. It has been shown that the same R factor leads to different phage restriction patterns in the three lysogenic strains of *E. coli* and in the two strains of *S. typhi-murium*. The possibility of using phage restriction patterns for the identification of R factors and *S. typhi-murium* strains of the same phage type but of different origin, is discussed.

BACTERIAL PLASMIDS AND THEIR IMPLICATION IN DIAGNOSIS

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Recently, a number of different bacterial plasmids has been characterized phenotypically and genetically. According to their somatic functions in the bacterial cell, plasmids can be classified into five groups, viz. (1) R plasmids, governing resistance of the bacteria to different chemotherapeutics; (2) virulence-plasmids, enhancing the virulence of the host or being responsible for the pathogenicity of the bacterial host by determining, e.g., enterotoxins, specific cell wall antigens or haemolysins; (3) sex-plasmids; (4) Col-plasmids; and (5) different types of plasmids ("taxonomic plasmids") governing various properties such as H₂S production or lactose fermentation, which form the basis of different diagnostic systems. The genetic and ecological observations concerning "taxonomic" plasmids have caused some confusion in diagnosis and epidemiology. The appearance of e.g. H₂S production in *Escherichia coli*, *Shigella sonnei* or *S. flexneri* as well as lactose fermentation in *Salmonella typhi* or other salmonellae leads to errors in routine diagnosis or to the missing of the

epidemic strain in question among other enteric bacteria. Restriction and amplification of the phage pattern by various plasmids may also lead to erroneous epidemiological interpretations. Bacteria of different phage types originating from a suspected epidemic focus were demonstrated to be due to plasmids (especially R plasmids) with restriction and amplification capacity. It seems reasonable to survey not only the transferable drug resistance but also the other plasmid-borne properties of the bacterial cell.

ELIMINATION OF INTEGRATED F FACTOR BY R PLASMIDS

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R fi^+ factors which completely suppress recombinant formation by *Escherichia coli* K12 Hfr strains were found in *Salmonella enteritidis*. The suppression was irreversible; acriflavine curing of the F factors failed to restore the donor character of the Hfrs.

UPTAKE OF DNA BY NEUROSPORA CRASSA

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The transformation of *Neurospora crassa* strains by DNA treatment was reported in 1972 by SZABÓ *et al.* at the 16th Annual Meeting on Microbial Transformation. The frequency of transformants was however, much lower than in the case of bacteria. To increase the frequency, the conditions favourable for DNA uptake were examined. ^{32}P labelled DNA was prepared from *Neurospora crassa* wild type (RL-3-8A) strain and added to *N. crassa* R-506-8-12 *inose*⁻, *rg*⁻ cultures in liquid medium. The uptake of DNA proved to be a two-stage process. In the first stage, DNA is absorbed in a DNase resistant form, not extractable by cold 0.5 M perchloric acid. The DNA adsorbed at a concentration of 10 $\mu\text{g/ml}$ DNA amounted to 0.2–0.4 $\mu\text{g/mg}$ dry weight of mycelium. In the second stage there was a continuous increase in uptake up to a 8–10-fold value of the amount initially adsorbed. DNA was isolated according to MARMUR. The rate of continuous DNA uptake depends upon the concentration of DNA and its degree of polymerization. There was no continuous uptake of DNA if the labelled DNA had been digested by DNase. If recipient cells were preincubated with highly polymerized (10 $\mu\text{g/ml}$) non-

radioactive DNA, the continuous uptake of labelled DNA (2 $\mu\text{g/ml}$) was inhibited, whereas the same concentration of digested DNA influenced the continuous uptake only slightly. DNA uptake is influenced by the conditions of cultivation, especially the composition of the incubation medium of the recipient strain. Maximum continuous uptake was found with young, log phase mycelia. There was no continuous uptake at 4°C or in the presence of 10^{-2} M sodium azide, 10^{-3} M EDTA, or at or above pH 7.25. Mg^{++} and Ca^{++} ions had a slight stimulating effect upon DNA uptake.

GENETIC BACKGROUND OF PORPHYRIN SYNTHESIS IN *BACILLUS SUBTILIS*

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Several hundreds of haematin dependent auxotrophs were isolated from cultures of *Bacillus subtilis* S168. The haematin-dependent strains could be divided into two groups: those replenishable with delta-amino laevulinic acid and those replenishable with haematin only. The former group was termed *haemA*. The strains replenishable with haematin only could be divided by a cross-transduction method into the further groups *haemB*, *haemE*, *haemC* and *haemD*. It was shown that *haemB* and *haemE* accumulated delta-amino laevulinic acid and their loci were transducible in linkage with *haemA*, while *haemC* and *haemD* did not accumulate delta-amino laevulinic acid and their markers showed no linkage with *haemA*. Attempts to determine the chromosomal location of the loci by transduction technique using phage 3NT have shown that *haemA*, *haemB* and *haemE* are located between the loci *leu-1* and *pheA* in the third segment of the *B. subtilis* chromosome. *HaemC* is located remote from the former ones next to the *argC* locus. The chromosomal location of *haemD* is not yet known.

KINETICS AND MECHANISM OF TRANSFECTION

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Earlier studies have shown that in the competent cells of *B. subtilis* 168 Ind⁻ infected with infective SP50 DNA, phage synthesis takes place on the cell membrane, probably on mesosomes transferred to the surface of the membrane in hypertonic milieu. The present results have supported these conclusions. The optimum conditions for transfection were modified. The

effectiveness of infection was increased by using for inoculation medium M-III instead of medium M-II used earlier. Under these conditions the kinetics and mechanism of transfection were investigated and the characteristics of the one-step reproduction cycle were determined. At the end of the one-step reproduction cycle, the number of virions attained the maximum in dependence on DNA concentration, competence, etc., without a lysis of most cells. In cultures infected with phage DNA, the number of cells was the same as in the control cultures and no products of bacterial lysis could be detected in the filtrate of the cultures by either spectrophotometry or chromatography or chemical methods.

PROTOPLAST FUSION OF AUXOTROPHIC GEOTRICHUM STRAINS

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Nutritionally deficient (*ade*⁻, *ileu*⁻, *lys*⁻, *met*⁻) stable mutants produced from *Geotrichum candidum* with nitrosoguanidine were converted into protoplast. Pairs of auxotrophic protoplasts were fused by close contact under various conditions, plated, and the plates were checked for complementation and protoplast regeneration. Using mannitol or glucose as stabilizer, complementations occurred at low but constant and reproducible frequency. At 20°C the average complementation rate was 8 per 5 million protoplast pairs at pH 5.5, and 3 per 5 million protoplast pairs at pH 7. At 4°C the frequency was lower. Colonies from complemented cells exhibited variable morphology. Fusion did not occur with KCl as osmotic stabilizer. No colonies appeared from separately treated protoplasts of the auxotrophic strains, if arthrospores or growing cells were mixed and plated as for protoplasts; if protoplasts mixed but not sedimented were plated; or if protoplasts of one strain were mixed with burst protoplasts from the other strain. Serial transfer could be performed on minimal medium indefinitely but these colonies produced mainly auxotrophic arthrospores. The proportion of segregation differed from colony to colony. A few complemented arthrospores always remained, but colonies from these again bore genetically split arthrospores, and stable complementation did not result. The complementation is ascribed to forced heterokaryon formation by protoplast fusion.

Virology

HISTOGENESIS OF THE ACUTE FORM OF MAREK'S DISEASE

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The development of histological lesions and the time of their appearance were studied in experimentally induced acute Marek's disease. In intraperitoneally inoculated animals, lesions manifested themselves in the liver and heart on the first day, more pronouncedly on the 2nd day in the wall of certain blood vessels by the proliferation of active mesenchymal (reticular) cells. On the 3rd day lymphoblasts, and on the 4th day foci consisting of mature lymphocytes appeared. From the 6th day on an increasing neoplastic proliferation of the lymphoreticular tissue was observed, especially in the liver and, later, in other extraneural organs. Lymphoid cells or neoplastic forms of reticulo-histiocytes were characteristic. After the 8th day, lymphoid cell vessel wall infiltrations and cuffings appeared around certain blood vessels in both the grey and white matter of the CNS. Lesions similar to those of the inoculated animals were observed in the same extraneural organs of naturally infected chickens 3-4 days later than in the artificially infected ones, but no changes appeared in the cerebrum and the spinal cord by the 13th day.

UNEXPECTED CLINICAL FORM AND SITE OF EQUINE COITAL EXANTHEM HERPESVIRUS REPLICATION

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Several equine herpesvirus species have been recognized, among them an agent responsible for the clinical syndrome termed equine coital exanthem. In the isolate 53/69 originating from a clinical outbreak of epigenital disease in Austrian horses was applied. In the first transmission experiment, viral takes were observed in each of 3 mares inoculated vaginally. One mare proved to shed the virus in her nasal discharge. Consequently 3 fowls aged 7 months were inoculated intranasally. Viral takes occurred in all the 3 fowls as shown by repeated re-isolations from nasal swabs of 3/3 animals, conjunctival swabs of 3/3 animals, and vaginal swabs of 2/2 animals. Lesions in the nostrils were clearly discernible, but the premature female genitals appeared to be unaffected. Papanicolaou smears from vagina and cervix of mares were rich in

bacteria and leukocytes, but without viral-nuclear inclusion bodies. The comparative virology of the new equine herpesvirus and the epidemiological significance of its nasal conjunctival shedding have been discussed.

RELATIONSHIP BETWEEN MAREK'S DISEASE AND THE BLOOD GROUP ANTIGENS IN TWO DIFFERENT CHICKEN LINES

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In two different chicken lines the relationship between Marek's disease and the blood group antigens A and B has been studied; 1500 birds were examined in each line. Half of each stock was vaccinated against Marek's disease at 1 day of age while the other half served as unvaccinated control. The whole stock was infected at 4 weeks of age with blood and organ homogenates obtained from chicken suffering from acute Marek's disease. Blood typing was carried out between 8 and 11 weeks of age in the whole stock. The surviving birds were sacrificed at the age of 13 weeks, and were, according to the post mortem data, classified in 3 groups *i.e.*, those which had died of Marek's disease and those with and without lesions of Marek's disease at sacrifice. Statistical evaluation of the post mortem data and blood typing of 2317 animals showed significant differences between the birds which did and those which did not possess B⁴ and B¹⁰ blood group antigens in the relation of resistance to Marek's disease within the two lines. The results were suggestive of a close relationship between B blood group antigens and immune response. This conclusion is in accordance with the fact that the B and C blood group systems similarly to the H-2 locus in the mouse, play an important role in the histocompatibility of chicken.

THE PATHOGENESIS OF RABIES

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Based on previous neuropathological investigations, the key importance of hypothalamic changes in the pathogenesis of rabies is emphasized. In the present study the route of Hőgyes's strain of rabies virus was followed from the periphery up to the hypothalamus. The spinal cord was transected and the virus was injected into the ischiadic nerve. The findings have elucidated the host-virus relationship from a new aspect.

MYCOPLASMA CONTAMINATION OF PRIMARY TISSUE CULTURES
PREPARED FROM BOVINE KIDNEYS AND TESTICLES

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Kidneys, testicles and sera of calves from various farms and primary tissue cultures prepared from the kidneys and testicles were tested for mycoplasma contamination. Altogether 24 mycoplasma strains were isolated. The strains obtained from the tissue cultures and sera belonged to *Mycoplasma arginini* and *Acholeplasma axanthum*. The same species as well as *A. laidlawii*, *A. modicum* and *M. bovirhinis* strains were isolated from lungs and peribronchial lymph nodes of calves. There was a close correlation between the mycoplasma contamination of the tissue cultures and the clinical picture of the disease observed in the corresponding farm, as well as between the quality of tissue cultures and the mycoplasma content of the corresponding organs, sera and tissue cultures.

REPLICATION OF HUMAN ADENOVIRUSES
OF DIFFERENT INTERFERON-INDUCING CAPACITY

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It has been shown earlier that the trypsinized crude adenovirus material, though fully infective, was not able to induce interferon production in chick fibroblast cells. This suggested the decisive role of a trypsin-sensitive protein in induction. Thus, the possible correlation between two capacities of human adenoviruses has been studied: (i) formation of non-viral paracrystalline structures during replication in HEp-2 cells, and (ii) induction of interferon in chick cells. HEp-2 cells infected with types 6, 8, 12 and 16, exhibiting different interferon-inducing capacity, were examined electron microscopically. No correlation was found between the two characteristics.

VIROLOGICAL EXAMINATION OF GYNAECOLOGICAL PATIENTS

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Attempts were made to isolate virus from endometrial scrapings and acute phase sera of 138 patients with menstrual disorders and from 16 control subjects. Fourteen adenovirus strains were isolated from the scrapings. Samples from 36 patients induced changes of focal array in primary human amnion cell cultures. Endometrial cells of 35 patients were studied with immunofluorescence method. Specific fluorescence referring to the presence of virus antigens was found in 23 cases, using herpesvirus and adenovirus antisera. Serological examination of the acute and convalescent sera of patients showed antibody titre rise only against type 2 herpesvirus and "latent" adenovirus types. Neither infective virus nor virus antigens were detected in the endometrial cells obtained from the cases of induced abortion serving as control.

EXPERIMENTAL DOUBLE INFECTION WITH TICK-BORNE ENCEPHALITIS
(TEV) AND HERPES SIMPLEX TYPE 1 (HSV) VIRUSES

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The incidence of inapparent and apparent human TEV infection and the number of reactors with HSV in various TE natural foci have suggested that mixed infections with both viruses are frequent. Studies in chick embryo cell cultures showed all possible ways of cooperation of both viruses in mixed infection: stimulation, inhibition and no mutual effect. In young cultures (used as model for rapidly renewing cells in the macroorganism) both viruses reproduced equally as, or better than, in single-infected controls. Their antigens were detected by immunofluorescence in different (for each virus) parts of polykaryocytes formed by superinfecting HSV from cells which had been yielding rich TEV progenium. In cultures aged *in vitro* (model for static cells in the organism) TEV interfered with the superinfecting HSV and interferon was stimulated by mixed infection. Experiments in adult mice double-infected intracerebrally proved the reproduction of both viruses in the brain. The incubation period of TEV was prolonged and shortened by previous and simultaneous HSV infection, respectively, retaining always its fatal course. Immuno-

fluorescence studies of double-infected suckling mice (highly sensitive hosts for both TEV and HSV) showed antigens of both viruses occupying different areas in the brain with minimum overlapping. The morphology of lesions was similar to that of single-infected controls. Mixed cerebral infection with TEV and HSV in rats (hosts of minimum susceptibility to both viruses) caused no increase in pathogenicity of any virus and 3-fold stimulation of interferon as compared with single-infected controls.

EFFECT OF SUPRAOPTIMAL TEMPERATURE ON CYTOMEGALOVIRUS MULTIPLICATION AND ON THE VIRUS INDUCED ANTIGENS

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Cell cultures were infected with cytomegalovirus (CMV) strain Rawles or AD-169 and incubated at 40°C. Cytopathic changes were observed. Infective virus could not be demonstrated in the disintegrated cells. The early intracellular antigens did appear, but late structure proteins could not be detected. Continuing the incubation at 37°C, all CMV-induced antigens appeared and virions were released. No difference in behaviour was found between the two strains under study at supraoptimal temperature. Both behaved similarly to HSV-2.

SEROLOGICAL DIAGNOSTICS OF INFECTIOUS HEPATITIS

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Indian ink grains were coated with commercial human gamma globulin and used in an agglutination reaction with serum samples from adult inpatients of the László Central Infectious Disease Hospital, Budapest. It was assumed that sera containing IH virus antigen (tentative designations IHx Ag) would agglutinate the coated Indian ink granules. Blood samples were taken at two-week intervals. Control group: 152 patients with obstructive or functional jaundice, toxic hepatitis or biliary disorders. In this group 10 patients carried hepatitis B antigen (HBAg) as shown by the micro-CF test, 15 were IHxAg positive and 3 proved to be positive in both tests; 122 cases (81%) were double-negative. Of the 105 patients diagnosed as IH on clinico-epidemiological basis, 11 proved to be HBAg positive, IHxAg negative, 19 double-negative, 2 consistently double-positive and 73 (70%) HBAg negative, IHxAg positive. Of the 118 cases diagnosed as SH, 45 (38%) were HBAg positive,

IHxAg negative, 30 (25%) were double-negative, 23 (19%) were HBAG negative IHxAg positive, 3 were of variable specificity, 14 were consistently double-positive. In each of 3 double-positive cases only one sample was available. Uncoated Indian ink grains were aggregated at low dilutions by all IH-Ag positive sera. In 60% of the cases, this non-specific reaction was of lower titre than the reaction with the gamma-globulin-coated reagent. Seventy chronic cases including 47 cases of hepatic cirrhosis, 16 cases of chronic persistent hepatitis and 7 cases of chronic aggressive hepatitis have also been examined. Of these, 16 cases were consistently IHxAg positive and 2 were HBAG positive and 5 were double negative. In the rest, eventual specific reactions were masked by anticomplementary effect and non-specific aggregation.

COMPOSITION OF ANTIGEN-ANTIBODY COMPLEXES IN VIRAL HEPATITIS

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Serum samples from 259 and 475 subjects having been exposed to hepatitis infection by the parenteral and oral route, respectively, were examined. The serum of 58% of them became anticomplementary up to a dilution of 1 : 8 to 1 : 128 in the second third of the incubation period; in 16.8% the anticomplementary activity was long-lasting. The immune complex in the blood of patients with hepatitis A contained antigen HBAG in 7%, hepatic tissue antigen in 5.5% and unidentifiable antigen in 88.5%. It is assumed that in about 55% of these cases DEL PRETE's IH antigen might have played a role in the complex. In the blood of the hepatitis B patients the complex contained HBAG in 85%, hepatic tissue antigen in 4% and unidentifiable antigen in 11%. Antisera to immune complexes were prepared in rabbits and guinea pigs. These reacted not only with the complex, but also with the human complement system as well as with human immunoglobulins.

HUMAN SLOW VIRUS INFECTIONS

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The slow virus infections of man are classified into three groups, viz., (1) inflammatory-viral type: subacute sclerosing panencephalitis (SSPE); (2) viral-encephalopathic type: progressive multifocal leucoencephalopathy (PML); (3) spongiform encephalopathies possibly viral in origin: Creutzfeldt-Jakob's disease (C.-J. d) and kuru. The slow virus infections are hetero-

geneous in their origin, epidemiology and clinical manifestation. They however, have in common that susceptibility to the infection and the clinical manifestation of the infection *viz.*, male majority in SSPE, age dependence in SSPE and C.-J. d. and tribal susceptibility in kuru, are determined, conditioned or at least influenced by genetic factors. The role of immune deficiency in the lymphatic apparatus, the pathogenesis of slow virus infections needs further research. The growing knowledge of slow virus infections makes it inevitable to revise the patho-morphological concept of inflammation and degeneration because the cellular alterations due to viral incorporation and multiplication have different consequences either inflammatory or purely degenerative in nature. The process of cellular inclusion production in SSPE and PML demonstrated by combined immunofluorescence, electron and light microscopic methods suggests that the same virus infection may manifest itself in different forms, *i.e.*, virus persistence, quiescence, cytolysis or even cell transformation.

SEROLOGICAL RESPONSE TO MEASLES IN SUBACUTE SCLEROSING PANENCEPHALITIS AND MULTIPLE SCLEROSIS

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Abnormalities of the cerebrospinal fluid (CSF) with regard to the occurrence of oligoclonal IgG and antibodies against different structural component of measles virus were studied in selected diseases. Matched serum and CSF samples were collected from patients with subacute sclerosing panencephalitis (SSPE) and multiple sclerosis (MS) and related to secondary optic neuritis and myelopathy of unknown cause. All patients with SSPE had oligoclonal IgG in their CSF and displayed a local production of measles antibodies in the central nervous system (CNS) as concluded from a comparison of the ratio of antibodies in serum and CSF to that of reference antibodies. In about half of all patients with MS, optic neuritis, and myelopathy with oligoclonal IgG in their CSF, a local production in the CNS of measles antibodies was also found. Further, a selective increase in serum titres of haemolysis-inhibiting antibodies was observed in patients with optic neuritis and myelopathy who had oligoclonal IgG in their CSF. The possible significance of activated latent virus infections inside and outside the CNS in the above-mentioned diseases was discussed.

PERSISTENT BORNA VIRUS INFECTION

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The pathogenesis of Borna disease was investigated by cultivating the virus in cell cultures and by a specific immunofluorescence technique. It was shown that Borna disease is a persistent, chronic virus infection of the central nervous system (CNS). Virus persistence was demonstrated in the CNS of horses (8 months), rabbits (12 months), hamsters (309 days), and in cell cultures (193 days). Sera from infected animals did not show CF antibody activity during the observation period. The possibility of vertical virus transmission under field conditions in horses is emphasized. The host spectrum of Borna virus is wide; it may extend to man. The persistent form of Borna virus infection in animals sheds new light on certain pathogenetic aspects of the disease. Borna disease might represent a useful model for studying persistent virus infections and slow virus diseases.

VIRUS-INDUCED AND REACTIVE ULTRASTRUCTURAL ALTERATIONS
IN CELLS OF THE CENTRAL NERVOUS SYSTEM

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Granular-surfaced tubules, similar to measles virus nucleocapsid, were found in every (4 biopsy and 1 autopsy) subacute sclerosing panencephalitis (SSPE) brain sample in the cytoplasm of oligodendroglia and vascular wall cells. Smooth tubules arranged in small bundles were seen in the nucleoplasm of oligodendroglia and nerve cells, and also in a nuclear pore of the latter. A pathogenetic significance is attributed to the fact that accumulated curved smooth tubules occurred only in the two brains which contained light-microscopic intranuclear inclusions. In the cultivated brain cells of SSPE patients and their co-cultures with a rhesus monkey kidney cell line viral components were absent although at some site the endoplasmic reticulum had transformed into a peculiar double spiral structure which, when accumulated, formed a cytoplasmic inclusion. It is suggested that the virus information content of the cells is responsible for their formation. In a brain sample from a progressive multifocal leucoencephalopathy patient, great numbers of polyoma virus particles occurred in the nucleoplasm of oligodendroglia cells; these cells dispaqued cytolysis. The spherical virus particles pass through the cell processes either into the myelin lamellae, or as a consequence of the cytolysis, on the surface of the lamellae, in extracellular position. In the nucleus of hypertrophied astrocytes, only few, and chiefly the elongated forms of virus, arranged

in small inclusions, were found. The cytoplasm, between the glia fibres and cytoplasmic organelles, contained granules with double envelope. This astrocytic transformation is ascribed to the presence of polyoma virus.

IMMUNOPATHOLOGY OF SUBACUTE SCLEROSING PANENCEPHALITIS
(SSPE)

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The serum anti-measles haemagglutination-inhibition (HI) titre may considerably fluctuate in the last six months of SSPE. In the occipital lobe, the immunofluorescent (IF) measles antigens showed little individual variation in intensity, whereas in the frontal lobe and in the cerebellum their variation was more intensive. A low serum HI titre was accompanied by a poor IF antigen content in the frontal lobe and the cerebellum. This was the case in a patient treated with azathioprine and another treated with dexamethasone.

PERSISTENCE OF FOOT-AND-MOUTH-DISEASE VIRUS IN SWINE

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Tissue fluids from organs of 20 pigs were examined for foot and mouth disease (FMD) virus. The animals in experiments 1 and 2 had undergone a serious type-C FMD epidemic 2–2.5 years before. No virus could be isolated from the samples collected at slaughtering. In experiment 3, five pigs were infected with FMD virus type C and slaughtered 26 to 64 days thereafter. Samples were taken from 22 parts of the body from each animal. In a pig slaughtered on the 26th day post infection, virus was demonstrated in the capsulae articulares metatarsae, in the thyroid gland and in the epiglottis. In a pig slaughtered on the 50th day, the tonsils and the epiglottis, and in one slaughtered on the 57th day an intestinal lymph node and the femoral bone marrow yielded virus. The remaining two pigs were slaughtered on the 64th day. Virus could be recovered from the tonsils of one of these.

INDUCTION OF THE INTEGRATED HERPES SIMPLEX VIRUS GENOME
IN HSV-2-TRANSFORMED HAMSTER CELLS

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Subcutaneous introduction of embryonic hamster fibroblasts transformed by HSV-2 cause tumour in hamsters. The *in vitro* cell line established from these tumours sheds no infective virus but 2–2.5% of the cells contain herpes-specific intracellular and surface antigens. In cells treated with IUDR an activation of the HSV genome took place. Seventy to 80% of the cells showed intracellular immunofluorescence with specific anti-HSV serum. Infective virus could not be recovered. Using arginine-less medium, 80% of the cells reacted with the antiHSV serum, but infective virus could not be recovered. The induced cells were of reduced oncogenicity *in vivo*.

TRANSFORMATION OF HUMAN EMBRYONIC LUNG CELLS
BY HERPES SIMPLEX VIRUS

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A productive HSV infection of primary human embryonic lung cells by different HSV strains was converted into a non-productive abortive latent infection. This was accomplished by incubating the HSV-infected cells at 42°C. The result was a transformed cell line in which a partially active genome was permanently present. The activity of this genome was proven by (a) syncytium formation as a phenomenon induced by viral genome activity; (b) positive virus-specific immunofluorescence in every cell; (c) occurrence of specific protein fractions resembling virus-induced proteins as investigated by acrylamide gel electrophoresis; and (d) unlimited growth of the cells *in vitro*. Whether or not these human cells are neoplastic will be investigated in immunotolerant mice. Investigation of viral and cellular DNA synthesis during incubation at 42°C revealed no excessive viral DNA synthesis and no virus production. In addition, cellular DNA synthesis, which is inhibited in non-infected cells at 42°C, persisted in the presence of the active viral genome. The results of these molecular studies have established the basis for the events involved in the herpesvirus-host interaction which may be the first step in neoplastic cell transformation by this virus.

ANTICELLULAR EFFECT OF INTERFERON

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In recent years data have accumulated which suggest that interferon (IF) preparations may exert also other than antiviral effects. Among these, for obvious reasons, the cell-growth-inhibitory effect (CGIE) is the most important. In the experiments reporting "non-viral" activity, interferons of various degree of purity were used. Sensitivity to the CGIE of IF varied with various cell-lines and cell-types. To gain more insight into the problem of action of IF on the cell, experiments were performed that showed that (a) the contact required for inhibition of L-cell multiplication by IF was about 4-8 times longer than the contact necessary for establishment of the antiviral resistance; (b) antiviral activity was found 30-50 times higher than the CGI capacity; (c) after prolonged passages *in vitro* primarily resistant mouse embryonic cells became sensitive against the CGIE of IF; (d) after prolonged passages *in vitro* in the presence of IF, an L-cell subline was obtained that retained its sensitivity to the antiviral effect of IF but was resistant against its CGIE; (e) in immunodiffusion tests with a specific anti-interferon globulin, precipitin lines were observed with both semipurified IF and deoxycholate-treated virus preparations; (f) electrophoretic fractionation failed to show a correlation between antiviral and CGIE activities. The results suggest that CGIE and antiviral activity are either two independent phenomena or the manifest or latent presence of a virus is a prerequisite of sensitivity to the effects of IF preparations.

CYCLIC AMP METABOLISM IN CELLS TRANSFORMED BY ONCOGENIC VIRUSES

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The distribution in different cytoplasmic membrane subfractions of adenylate cyclase and cyclic-AMP phosphodiesterase was investigated in cells transformed by SV-40, Rauscher leukaemia virus or HSV-2, as well as in supernatants free of cell particles, prepared by centrifugation at 105 000 g_{av} . The results were compared with values for non-transformed cells. The supernatants from all transformed cells contained adenyl cyclase which was sensitive to hormone and fluoride and insensitive to prostaglandins. Only traces of this enzyme were present in the cell fractions of normal cells. From the transformed cells three, from the corresponding normal cell two, cytoplasmic membrane

subfractions were obtained. They were characterized by electron microscopy and marker enzymes. Hormone-sensitive adenyl cyclase (sucrose density, 1.16) was demonstrable in each of the three subfractions of the transformed cells, whereas in only one of the normal cells. Sensitivity of the adenylate cyclase to adrenaline and glucagon and to stimulation with prostaglandins depended on the transforming virus (RNA or DNA virus). The cyclic-AMP phosphodiesterase occurs mainly as a soluble enzyme, but can be detected in all-membrane subfractions obtained from both normal and transformed cells. The adenylate cyclase/cyclic AMP phosphodiesterase ratio depends on the transforming agent (RNA or DNA virus).

THE SUPPRESSIVE EFFECT OF NDV ON THE PRIMARY ANTIBODY RESPONSE OF HAMSTERS

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A correlation has been suggested between the immunosuppressive and interferon-inducing effects of human adenoviruses in chickens. In this work another virus-host system was applied. Since NDV is known to be a good interferon inducer in hamsters, its effect on the primary immune response to sheep red blood cells was studied. A single intraperitoneal injection of NDV (5×10^8 p.f.u.) markedly depressed the 19S haemolytic plaque-forming cell response in the spleen to immunization with sheep red blood cells. The immunosuppressive effect of NDV depended on the interval between the administration of the virus and of sheep red blood cells, similar as in adenovirus infected chickens. These data support the authors' previous assumption of a relationship between the interferon inductive and immunosuppressive capacities of a virus.

AGE DEPENDENT CHANGES IN THE CELLULAR IMMUNE REACTION OF MICE TO LYMPHOCYTIC CHORIOMENINGITIS VIRUS INFECTION

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The cellular immune response to lymphocytic choriomeningitis (LCM) virus infection was studied in C3H, A and C57BL mice of different ages. Six to 10 weeks and 8–24 months old mice of different strains were infected intracerebrally with 100 ID₅₀ of LCM virus simultaneously. All the 6 to 100 weeks old mice succumbed 6–8 days after infection, displaying the characteristic

symptoms and histological picture of LCM. The course of infection was different in 8–24 months old mice: death occurred between the 3rd and 12th day after infection and only part of the animals showed symptoms characteristic of LCM with meningeal infiltration. Similar changes were previously found in the course of LCM virus infection in animals whose ability to develop cellular immune response was reduced artificially. The present results revealed a reduced cellular immune response in 8–24 months old mice. Studying the lymphoid system of these animals, thymic involution due to age was found to be responsible for the weakness of the cellular immune response.

THE DYNAMICS OF HEPATITIS B ANTIGENAEMIA AND ANTIBODY TITRE
FROM EARLY INCUBATION TO LATE CONVALESCENCE

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In 1969–1971, a total of 259 persons exposed to parenteral hepatitis B infection were followed up from the time of exposure to late convalescence (*viz.*, for three months after clinical recovery). Serum samples were tested at 3–4 week intervals during incubation, weekly in the clinical period and monthly during convalescence. Of the persons studied, 187 became sick, 72 remained healthy. (The latter were observed for 200–210 days after exposure.) Detectable amounts of hepatitis B antigen (HB_{Ag}) had appeared in those who became sick as early as in the second half of the incubation period, thus preceding the increase in serum transaminase activity by 10 days on the average. In the last week of the clinical period, 8.5% of these patients were still positive. Antibodies showed the maximum level in the 4th week after onset of disease. Persons remaining healthy exhibited a transient HB_{Ag} positivity in the first week of incubation, followed by a transient HB antibody positivity in 83%, indicating that they had already been exposed to HB_{Ag}.

POTENCY CONTROL OF FOOT-AND-MOUTH-DISEASE VACCINE
IN ADULT MICE

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A method of potency test for mono and trivalent FMD vaccines was developed in inbred mice of 8–10 weeks. Each lot of vaccine was tested in 25 mice divided into five equal groups. The animals were vaccinated subcutaneously with five different dilutions of the vaccine *viz.*, 1 : 1, 1 : 2, 1 : 4,

1 : 8 and 1 : 16. The individual dose was 0.7 ml when monovalent and 2.1 ml when trivalent vaccine was tested. PBS was used as diluent. Each animal was challenged intraperitoneally with 10^4 ID₅₀ of mouse-adapted FMD virus. All the control animals died within 48 hr, showing specific symptoms. The 50% mouse-protective dose (MPD₅₀) was calculated for each vaccine. Since different inbred mouse stocks reacted differently both to vaccination and to challenge, the same was used throughout. A challenge dose of 10^4 LD₅₀ proved to be the most suitable. On the evidence of the repeated control of 3 vaccines, reproducibility of the results was considered satisfactory. For the determination of the limit values in vaccine qualification, further comparative examinations are required.

JOINT ACTION OF DNA AND RNA VIRUSES IN THE DEVELOPMENT OF MALIGNANT TUMOURS

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A papovavirus of golden hamster producing epithelial skin tumours, which was administered to newborn hamsters of a strain free of spontaneous tumours, yielded a high percentage filterable leukoses as well as cell-free transmissible sarcomas and, additionally, mesotheliomas that occurred almost exclusively in female animals. In Wistar rats the virus induced reticulosis. In these four mesenchymal tumours, in contrast to hamster skin tumours, the papovavirus was no longer detectable but, instead, a C-type RNA virus has been observed regularly. It was possible to produce leukosis also with the isolated circular DNA of hamster papovavirus (molecular weight, 3.6×10^6). The "inducer effect" of the papovavirus is explained in terms of an activation a latent hamster or rat C-type virus inducing leukosis, sarcoma, mesothelioma and reticulosis in the inoculated animals. This suggestion is supported by simultaneous electron microscopic detection of both papova and C-type viruses in thymus cells a few days following infection of newborn hamsters. One component of the C-type virus activation appears to be an immunodepression brought about by thymus lesion, because thymectomy in the hamster strain used also leads to a certain formation of leukaemia and mesothelioma. Application in the same hamster strain of cell-free filtrates from different human malignomas also induced a significant percentage leukosis the origin of which has not been clarified. In some of these human tumours, C-type particles or particles suspicious of virus were demonstrated.

EFFECT OF EPSTEIN-BARR VIRUS ON DNA SYNTHESIS IN LEUCOCYTES

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A cellular-DNA-stimulating effect is considered characteristic of DNA tumour viruses. Purified white-blood-cell suspensions obtained from healthy subjects and lymphoid leukaemia patients were infected with EBV. In the infected cell suspensions, DNA synthesis was measured by radioactive thymidine incorporation (10^7 cells/ml; Parker's 199 with 10% calf serum; 37°C; 5% CO₂; 2.5 μ Ci ³H/ml sample; 24 hr). DNA synthesis in the cells from normal subjects was slightly stimulated by the infection while it was intensely stimulated in lymphocytes from patients with chronic lymphoid leukaemia and left unaffected in cases of chronic myeloid leukaemia. Inactivated EBV, cytomegalovirus or HSV-2 failed to induce DNA synthesis. No viral antigen could be detected in the induced white blood cells. The induced DNA synthesis was evenly increasing from the 4th day post infection to the end of the experiment on about the 12th day. Of the white blood cells, B lymphocytes seemed to be the most sensitive to EBV.

LIPIDES IN THE SPLEEN OF BALB/C MICE INFECTED
WITH RAUSCHER VIRUS

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The lipid content of the spleen of Balb/c mice undergoes changes during the development of leukaemia. The amount of neutral triglycerides decreased by 44%, whereas that of the free fatty acids increased by 70%. Changes were observed in the composition of the phospholipides: lecithine, phosphatidylethanolamine and phosphatidyl-inositol increased, cardiolipin and sphingomyelin decreased in amount. Phospholipide synthesis was examined by measuring ³²P incorporation into phospholipides. This was 8–10 times higher in normal animals than in mice with leukaemia.

IMMUNOCHEMICAL STUDY OF THE SOLUBLE ANTIGENS AND ANTIGEN-
ANTIBODY COMPLEXES IN THE SERUM OF MICE WITH RAUSCHER
LEUKAEMIA

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In the serum freed from virus of mice with Rauscher leukaemia, two soluble antigens are detectable: one of α_2 and another of beta electrophoretic mobility. The same antigens could be detected by indirect immunofluorescence in cell membranes of Rauscher leukaemia cells. The concentration of the antigens in serum is in direct relation to the progress of leukaemia. Under natural conditions mice absolutely or relatively resistant to the Rauscher leukaemia virus produce antibodies only to the antigen migrating in the α_2 globulin fraction. The component of beta electrophoretic mobility needs complete Freund adjuvant to induce an immune response. The antigen of α_2 mobility forms a complex with the corresponding antibody and thus reduces the cytotoxic activity of the immune serum. The antigen-antibody complex dissociates at low pH and the antigen can be freed from antibody by gel filtration.

GENETIC BACKGROUND, MECHANISM AND PATHOGENETIC ROLE
OF IMMUNOSUPPRESSION INDUCED BY THE RAUSCHER VIRUS COMPLEX

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In inbred Balb/c, DBA/1 and C57B1/10Sn mice and their F1 hybrids and F2 generations, sensitivity to Rauscher virus is determined by two genes, viz., by the R-2 locus in which the allele-determining sensitivity is dominant, the malignant transformation is controlled, whereas locus R-1, in which the allele responsible for resistance is dominant, influences the course of the leukaemia. In the Rv-1^s-Rv-2^s Balb/c and in the Rv-1^R-Rv-2^r C57B1/10Sn mice the course of the disease was not modified by pretreatment with heterologous ALS, whereas in the moderately susceptible DBA/1 mice of genotype Rv-1^R-Rv-2^s the progress of leukaemia was considerably accelerated. Balb/c mice with Rauscher leukaemia gave hardly any tumour-specific antibody response. The cellular immune response in DBA/1 mice was as strong as in the C57B1/10Sn mice, but the former animals produced less tumour-specific antibody. Immunosuppression ran parallel with virus multiplication in the lymph nodes. Since it is not the gene responsible for malignant transformation that controls the immunosuppressive effect of Rauscher virus (the malignant transformation may be attributed to the virus multiplying in immuno-com-

petent cells), the effect of the Rv-1 gene is thought to be expressed through the sensitivity to the helper virus of the Rauscher virus complex and/or through determining the resistance.

MORPHOLOGY OF MC-29 VIRUS-INDUCED LIVER TUMOUR IN CHICKEN

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Primary hepatic growths induced by administration of a variety of carcinogens have attracted attention for many years. Until recently there was no implication of viruses in the induction of primary neoplasms in the liver. It was therefore of much interest that according to observations made in Dr. J. W. BEARD's laboratory (Durham, N.C., U.S.A.), one of the avian leukosis viruses, the MC-29 strain isolated in Sofia, was able to induce primary liver tumours in a high percentage of chickens infected intravenously one day after hatching. The light and electron microscopic features as well as the histogenesis of these virus-induced primary liver tumours are reported and their characteristics discussed in comparison with those of chemically induced experimental hepatomas as well as with those of human liver tumours.

TRANSPLANTABLE HEPATOMA ESTABLISHED FROM PRIMARY LIVER TUMOURS INDUCED BY MC-29 VIRUS

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Intravenous injection of MC-29 RNA virus into 1 to 3 day old Duke white Leghorn chicken induces primary liver tumours in 40–50% of the birds. Introducing small pieces of these primary tumours intraperitoneally or intramuscularly into newly hatched chicken, solid tumours weighing about one quarter of the total body weight developed within two weeks. Transplantable hepatoma lines were established by further passages. Histological examination of the transplanted tumours of the first 2–3 passages revealed the picture of malignant liver tumours of sometimes glandular, but mostly trabecular, structure. The tumours in the later passages proved to be poor in stroma and had an exclusively trabecular character. The tumour cell is characterized by an enlarged nucleolus (sometimes more than one). Ultrastructural features of liver cells including the presence of biliary capillaries could be identified by electron microscopy of the tumour cells. The presence of virus in the intracellular spaces and biliary capillaries was also often observed. From the 7th

passage of the intraperitoneal line established in Durham, subcutaneous and intramuscular lines have been formed in Hungary in Hunnia hybrid chickens. In May 1973, the 12th passages were performed. After transplantation, 80–100% of the animals developed local tumours weighing about one quarter of the total body weight; 80–100% of the animals died within 20 days. The transplantable hepatoma reported represents the only experimental transplantable hepatoma of viral origin and is therefore suitable for comparative studies.

CELL POPULATION KINETIC PROPERTIES OF A CELL LINE
OBTAINED FROM A VIRUS-INDUCED CHICKEN HEPATOMA

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The virus strain MC-29 induces in the chicken different types of haemoblastoses and hepatomas. Transplantation of one of the hepatomas resulted in the establishment of an *in vivo* transplantable hepatoma line. LAPIS and LANGLOIS produced from cells of this line an *in vitro* cell culture, which in Hungary had been passaged 42 times by June 1973. The hepatoma cells are cultured as a monolayer in Falcon dishes using Medium 199 containing 20% foetal calf serum. A suitable cell number for passage was 200 000/ml medium. Twenty-four hr after the passage, about 50% of the cells attached to the surface of the dish. Between the 24th and 48th hr and also between the 48th and 72nd hr doubling of the cell number was observed, but later the proliferation slowed down. The ratio of mitotic figures was 1.5% at 48 hr, 1.8% at 72 hr, and 0.6% at 96 hr. On labelling with [³H]thymidine, the labelling index was 35.6% at 24 hr, 48.5% at 48 hr and 25.7% at 72 hr. Autoradiographic and cytophotometric studies of the cell cycle time and cell cycle phases are reported.

ADAPTATION TO DUCK EMBRYO OF INFECTIOUS BRONCHITIS VIRUS,
NEWCASTLE DISEASE VIRUS AND FOWLPOX VIRUS
FOR DEVELOPING LIVE VACCINES

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The fowl pseudopest vaccine strain La Sota, the fowl strain of fowlpox and the infectious bronchitis vaccine strain Massachusetts have been adapted to the duck embryo. The duck embryo was chosen because the duck is not sensitive to the most dangerous viral infections and tumours communicable

to fowl. In the first passage, the La Sota strain yielded a one logarithm lower haemagglutination titre in the duck embryo than in the chick embryo. In subsequent passages the titre increased and in the eighth duck embryo passage it reached a value similar to that in chick embryos. Fowlpox virus was inoculated onto the chorioallantoic membrane. An oedema of membranes, and nodules occurred only up to a dilution of 10^{-3} . In the chick embryo similar changes appeared up to the 10^{-6} dilution. In passaging strain Massachusetts the infective and serum-neutralizing titres showed great variations. For this reason the experiments are continued with different methods.

IMMUNODIFFUSION REACTION WITH ANIMAL INFLUENZA VIRUSES AND ANTIBODY

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The gel double diffusion chamber microtechnique has been used for immunodiffusion (ID) studies of various animal anti-influenza sera with influenza A virion antigens. With rabbit and guinea pig hyperimmune sera or human convalescent sera, highly concentrated virus antigens are usually necessary for a positive gel precipitin reaction, an excess of antigen(s) being sometimes desirable for the detection of low amounts of antibody. Several peculiarities have been encountered in ID tests with swine and horse sera containing antibody to influenza virus subunits. When working with these sera, the antigen must be applied at proper concentrations. An excess may lead to a failure of precipitin formation and thus to erroneous results. Estimation of proper antigen contents by ID titration is of partial value only: first, different amounts of disintegrated virions are optimal for the detection of individual influenza virus subunits and, second, the optimum amount of virus antigen as found with one serum need not necessarily be optimal for antibody detection by the ID test in another serum, although both sera may show the same antibody titres in haemagglutination-inhibition or neuraminidase-inhibition tests. With various, though closely related, influenza virus strains as well as various modifications of the ID technique, different precipitin patterns are obtained. The same holds for disintegration of virions with different detergents. These phenomena often appear more marked in ID tests with swine "field" and horse anti-influenza sera than with human convalescent or rabbit and guinea pig anti-influenza sera.

ACUTE RESPIRATORY VIRUS INFECTIONS IN NORTH CROATIA
FROM 1962 TO 1971

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In the period 1962 to 1971, 37 350 sera taken from 8438 patients with respiratory infections were analyzed serologically for the presence of CF antibodies for influenza A and B viruses, parainfluenza viruses, respiratory syncytial (RS) virus, adenoviruses, *Coxiella burneti*, *Bedsonia* (ornithosis), and *Mycoplasma pneumoniae*. On the basis of the referral clinical diagnosis the patients were divided into 3 groups: group A, pneumonia (2127 patients); group B, other respiratory conditions (4839 patients); and group C, no clinical diagnosis (1472 patients). The frequency of CF antibodies to the above mentioned agents was 27.4% in group A, 24.7% in group B, and 20.6% in group C. Thus, most agents produced just as many pneumonias as they did, other respiratory conditions, whereas *Bedsonia* (ornithosis), *C. burneti*, and *M. pneumoniae* were more frequent causes of pneumonia than of other respiratory conditions. As regards age, the lowest frequency of influenza occurred in young age especially in infants under one year of age. Parainfluenza, RS, and adenovirus infections increased in frequency up to the 7th year of life but a considerable number of parainfluenza and adenovirus infections was observed in older age groups. The higher proportion of *C. burneti* and *Bedsonia* in the 20 to 59 year age groups may be explained by occupational exposure. The seasonal incidence of different viruses over 10 years did not differ significantly from the seasonal occurrence of the corresponding respiratory infections in Croatia.

ANTIGENIC ANALYSIS OF INTERPANDEMIC INFLUENZA VIRUS STRAINS

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During the interpandemic "antigenic drift" of influenza A virus, some antigenic determinants of the viral haemagglutinin disappear and are replaced by others. Thus, some of the determinants in two strains are common (c) others are regressive (r) or progressive (p) determinants, present in one of the strains only. The cross HI test distinguishes between strains on the basis of the quantity of antibody reacting with the "c" determinants. In the serum absorption test developed by the authors, differentiation is based on the antibodies reacting with the "r" or "p" determinant but not with the "c" determinant. By means of the method interpandemic strains can be ranked in a "progression gradient" reflecting the antigenic drift taking place in the interpandemic history of human influenza A virus.

PRACTICAL VALUE OF THE "PROGRESSION GRADIENT"

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Using the serum absorption test evolved by the authors, "monospecific" influenza antisera have been prepared. These are suitable for rapid identification of newly isolated influenza A virus strains on the basis of their position on the "progression gradient". Besides, it can be decided whether the strain was introduced from other areas or came into being locally as a result of strain variation. The greatest practical value of the progression gradient lies in its use for the selection of vaccine strains.

TISSUE SPECIFICITY AND GROWTH PATTERNS OF INFLUENZA VIRUS
IN FERRET AND HUMAN ORGAN CULTURES

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A comprehensive survey of ferret tissues in organ culture showed that influenza (A/H2N2/Moscow/1019/65) can infect the uterus, oviduct, urinary bladder and conjunctiva in addition to respiratory tissues. Alimentary tract tissues were not susceptible except the oesophagus which was successfully infected with large inocula. Blood vessels, spleen, lymph nodes, muscle and kidney were not susceptible. No differences could be detected between tissues in their capacity to adsorb virus. Minimal infectious dose determinations on susceptible tissues showed great variations in susceptibility. The most susceptible nasal turbinates were followed by the bladder, uterus, trachea, lungs and the oesophagus. The growth pattern of the virus determined by taking samples every 24 hr for a period of 4 days showed different kinetics in different tissues. These differences were abolished or considerably reduced on increasing the inoculum. On the other hand, the kinetics of virus replication over one infection cycle were similar for all susceptible tissues. Experiments with human organ cultures indicated that the human tissue specificity of the virus is not restricted to the respiratory organs. The length of the one-step growth cycle in the human endometrium was similar to that observed in ferret tissues.

IMMUNOFLUORESCENCE AND ELECTRON MICROSCOPY OF RS VIRUS
INFECTED HeLa CELLS

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The appearance of new virus antigen was investigated in HeLa cells inoculated with the Long strain of RS virus. Ten hr after the infection, the new virus antigen could be detected in the cytoplasm as a fine point-like fluorescence. After 14 to 18 hr the number and extension of the specifically glittering spots increased and after 20 to 24 hr almost the whole cytoplasm displayed specific immunofluorescence. In some cultures between 10 and 14 hr one, occasionally two, sharply demarcated inclusions appeared in the cytoplasm; the inclusions were surrounded by a clear halo which showed intensive immunofluorescence. In preparations stained with methyl green-pyronine, the greater part of the inclusions was green (DNA?) in the pyroninophilic cytoplasm. Electron microscopically the inclusions composed of fine granular dense structures were surrounded in the place of the halo with nucleocapsid-like structures resembling those described for paramyxoviruses. Similar structures were observed also in other parts of the cytoplasm of these cells.

POOR EFFECTIVENESS OF LIVE POLIOVIRUS VACCINE
IN TROPICAL COUNTRIESI. DÖMÖK, M. S. BALAYAN, O. A. FAYINKA, A. D. SONEJI, NADA SKRTIC
and E. HARLAND*WHO Virus Study Team, Entebbe and MRC Child Nutrition Unit, Kampala, Uganda*

A virologically controlled field trial with type 1 live poliovirus vaccine was carried out on children between 3 and 36 months of age living in a rural area of Uganda. The children were divided into four groups. Groups A and B were composed of breast-fed children, whilst groups C and D of children who had no milk whatever. Type 1 vaccine (10^6 TCID₅₀ dose) and 0.5 ml anti-human gamma globulin horse serum were given orally to the children in groups A and C, while only the vaccine was given to those in groups B and D. Sero-conversion rate proved to be 72% (42/58) in group A, 56% (33/59) in group B, 72% (26/36) in group C, and 46% (13/28) in group D. Similar results were obtained when the rate of vaccine virus excretors was investigated. Thus, children who had received the vaccine together with anti-human gamma globulin responded significantly better than those who had received the vaccine alone, irrespective of whether they were breast-fed or fed without milk at the time of vaccination. The presence or absence of enteroviruses in the gut

of children at the time of vaccination also influenced the results. Seroconversion rate in groups A + C was 67% when enteroviruses were present and 78% when they were absent. The same rates in groups B + D were 45% and 59%, respectively. The results clearly indicate that (i) breast-feeding does not influence the effectiveness of the vaccine in the age groups tested; (ii) an inhibitor must be present in African children decreasing the rate of vaccine virus implantation and consequently the serological response, which can be blocked to a certain extent by anti-human gamma globulin; (iii) in contrast to certain observations, interference between enteroviruses and the vaccine strain also plays a role in decreasing the vaccine's efficacy. Vaccine virus excretion was usually of short duration and the antibody titres acquired either by the vaccination or by natural infection were unusually low. These phenomena also seem to be due to the presence of poliovirus inhibitor in African children. There was no significant difference between children having naturally or artificially acquired immunity in respect of intestinal resistance to reinfection. In the course of this trial lasting about 9 months, from 2274 stool samples 796 strains belonging to 40 different types of enteroviruses pathogenic to suckling mice were isolated.

ISOLATION OF POLIOVIRUS TYPE 1 AND TYPE 3 FROM THE NASOPHARYNX
OF HEALTHY CHILDREN AND CHILDREN WITH RESPIRATORY DISEASE

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From 306 samples of nasopharyngeal secretions, collected in May-July, 1970 from 0-5 years old children apparently healthy or with mild upper respiratory diseases, 84 viral agents including 12 polioviruses were isolated. The isolations were done 7-90 days after primary or revaccination against poliomyelitis. Of the isolated poliovirus strains, 10 belonged to serotype 1, exhibiting the characters of attenuated virus and 2 to serotype 3, having wild strain character. In 1971, from 20 infants with lower respiratory tract disease, 20 poliovirus type 3 strains were isolated, which showed intermediate ret_{49} character. The possibility of poliovirus isolation from the nasopharynx and the possible involvement of these viruses in the aetiology of some upper and lower respiratory tract diseases, have been discussed.

EFFECT OF ANTISERUM ON GUANIDINE-INHIBITED INTRACELLULAR
POLIOVIRUS

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Monolayers of permanent monkey kidney cells were infected with poliovirus. The survival of infective centres was determined following treatment of monolayers with different chemical inhibitors and antisera. Intracellular poliovirus was shown to be neutralized by type-specific antisera added to the maintenance medium of infected cells, if intracellular viral processes were restricted by guanidine during antiserum treatment. Penetration of antibodies through the membrane of infected cells seems to offer the most plausible explanation of the phenomenon. Guanidine was shown not to be responsible for the intracellular neutralization observed. The mechanisms which might be involved in the phenomenon have been discussed.

SERUM-NEUTRALIZING ANTIBODIES TO POLIO AND COXSACKIE
A9 VIRUSES COLLECTED ON FILTER PAPER DISKS

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To test samples collected from TPNG, whole blood drawn from the finger-tip was let to absorb by filter paper squares; after drying, these were sent to the virus laboratory which was about 8000 miles away. In the laboratory, the papers were eluted and distributed in four tubes according to the 4 serotypes of virus. More than 1200 samples were tested. (i) The polio antibody level varied from area to area. Of seven differently isolated areas, two showed a low incidence of polio type 1 antibody (1 and 8%). Polio type 2 antibody was more frequent (33–77%) than type 3 antibody (30–50%). The incidence of coxsackie A9 antibody was high (37–78%). (ii) Investigations in vaccinated and non-vaccinated areas have shown that — apart from Sabin's type 2 polio vaccine — polio vaccination did not cause any dramatic change in the frequency of antibody. (iii) The new-borns' antibody level was usually equal to, or lower than, their mother's antibody level.

Immunology

COMPARATIVE IMMUNOLOGICAL AND IMMUNOMORPHOLOGICAL STUDIES ON PROTECTIVE ANTIGENS OF BACILLUS ANTHRACIS

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The protective antigen of *Bacillus anthracis* was prepared, purified, and characterized immunochemically and immunomorphologically. The immunomorphological events ensuing in the regional lymph nodes and parenchymal organs of animals pretreated with the protective antigen were followed up histologically. Animals immunized with live CTI-1 anthrax vaccine served as controls.

IS THE RESISTANCE TO SALMONELLA INFECTIONS OF MICE DUE TO CELLULAR IMMUNITY?

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The views are surveyed which have served as basis for some investigators to attribute the immunity of mice against salmonella infections to cell-mediated immunity. An account is given of immunological experiments including passive serum treatment, active immunization, and the mucin technique, investigations with macrophages, and studies on antiendotoxic immunity. The results showed that specific antibodies appearing in the sera of treated mice play an essential role in the immunity of these animals against infection. Immunity of mice against salmonella infection is a typical example of the immunity wherein humoral antibodies and phagocytes are functioning jointly.

IMMUNITY IN HUMAN SALMONELLOSES

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It has been shown that salmonella infection results in well-measurable immunological and serological changes. Those in the haemagglutination titre of sera are considered of diagnostic importance. The dynamics of the increase and those of the decrease in the protective titres suggest that the immunity

developing after infection lasts for a short period. Furthermore, the changes induced by infection in the titres of bactericidal antibodies show that this type of the immune response must also be reckoned with. Since no similar responses have been observed after a single uptake of a high amount of salmonellae or endotoxin, it is suggested that the pathological symptoms of salmonella infection and the responses serving protection do not develop unless living bacteria enter the intestinal wall.

NEW METHOD FOR THE IMMUNOBIOLOGICAL STANDARDIZATION OF TUBERCULIN

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The Hungarian Standard PPD was developed more than 15 years ago. The 6th Hungarian Pharmacopoeia and the production technology prescribe the potency testing of tuberculin in guinea pigs, but requirements of immunobiological standardization are ambiguous. Up to now the potency of the standard and test preparations have been compared by a simple arithmetical summarization of the sizes of the 24 and 48 hr skin reactions in guinea pigs. Using the data obtained by testing the Hungarian Standard PPD in one hundred guinea pigs during the last two years, the dose-response diagram of the standard preparation was determined. This makes it possible to read the potency of a test preparation directly in per cent compared with the standard material and to estimate the standard deviation of a given test.

STANDARDIZATION OF TOXICITY TESTING FOR COMBINED VACCINES (DTP)

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The mouse-toxicity test (so-called weight-gain test) for diphtheria-tetanus-pertussis vaccines was carried out according to the Requirements for Pertussis Vaccine of the WHO. Mouse toxicity of the vaccines is mainly caused by the early appearing toxicity of the aluminium phosphate adsorbent and the early and late toxicities of the pertussis component. After the injection of a combined vaccine, the weight-gain of mice is less than it would be caused by the injection of the adsorbent and/or pertussis component of a given toxicity. The regression lines evaluated from the data are better characterizing the weight changes of mice injected with different vaccines than does the absolute or relative weight-gain on the 7th day after injection according to

the different international requirements. The aluminium phosphate adsorbent significantly increased the late toxicity of the pertussis component. The toxoids have similar but slighter effects. Diphtheria toxoid in high doses has a significant early toxicity. Tetanus toxoid in high doses has a similar but slighter effect. The toxoids do not exert late toxicity.

OCCURRENCE OF "NATURAL" VIBRIOCIDAL ANTIBODIES IN THE POPULATION OF A NON-ENDEMIC AREA

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The sera of 334 healthy Hungarian blood donors who have never stayed in an endemic area and have never been given anticholera vaccination were investigated for "natural" vibriocidal antibodies. Vibriocidal antibodies were found in 45.3% of the sera. There was no sex difference in this respect. Pooled serum made of antibody positive sera had a considerable mouse-protective capacity, while the pooled negative sera had no such property. No difference in the distribution of "natural" vibriocidal antibodies was found among the various ABO blood groups; nor was it possible to show any difference between the Rh-positive and Rh-negative groups. Finally, the antigenic relationship to which the presence of the "natural" vibriocidal antibodies can probably be ascribed, is discussed.

IMMUNOLOGY AND PUTATIVE VIRAL AETIOLOGY OF HUMAN SARCOMAS

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Investigation of the possible viral aetiology of human sarcomas consists of the following experimental approaches: (i) Establishment of tissue cultures of human sarcomas. In our laboratory, 14 human sarcomas cell lines were established as permanent cultures. (ii) Demonstration of sarcoma-specific antigens in sarcoma cells. Using the indirect immunofluorescence technique and sera of sarcoma-bearing patients, two series of studies were done. In the first series done with Dr. E. SHIRATO, it was shown that 13 out of 22 sarcoma patients had antibodies directed to autologous tumour cells (59%); in eight normal sera, no such antibodies occurred. In the second series done with Dr. F. GONZALEZ, 24 of 88 sera collected from sarcoma patients (27%) reacted with sarcoma cells. Sarcoma-specific antigens were also detectable by a lymphocyte-mediated cytotoxicity assay. In a study done with Dr. L. CAMPOS and

Mr. J. CABINESS, 92 out of 126 (73%) sarcoma-bearing patients yielded lymphocytes that destroyed cultured sarcoma cells preferentially to cultured carcinoma cells. In another study done with Dr. H. D. KAY, the purified lymphocytes of sarcoma patients destroyed cultured sarcoma cells preferentially to carcinoma cells but some normal healthy individuals also yielded lymphocytes with similar activities. Serum factors antagonistic to or synergistic with cytotoxic lymphocytes occurred frequently in the blood of sarcoma-bearing patients and occasionally also in the blood of normal individuals. (iii) Antigenic conversion *i.e.*, the expression of sarcoma-specific antigens in normal human embryonic cell cultures after exposure to cell-free sarcoma extracts or culture fluids. In a study done with Dr. E. SHIRATO, a chondrosarcoma cell line yielded cell-free supernatant fluids capable of causing antigenic conversion in three out of ten attempts in normal human embryonic cells. In another study done with Dr. F. GONZALEZ and Miss S. SCHNEIDER, eight sarcoma cultures yielded cell-free fluids capable of causing antigenic conversion of normal human embryonic fibroblast cultures in 374 experiments, varying from culture to culture from 9 to 21% of the attempts. (iv) Focus formation, *i.e.*, neoplastic transformation of normal human embryonic cells after exposure to cell-free fluids from sarcoma cultures. In one study, cell-free fluids from eight sarcoma cultures caused focus formation on two occasions out of 37 tests. In another study done with Dr. F. GONZALEZ and Miss S. SCHNEIDER, in 26 experiments focus formation occurred only twice. (v) Demonstration of virions, subviral structures or viral genome fragments in sarcoma cells. Type C virions were seen only occasionally in sarcoma cells but filamentous structures resembling, but not entirely identical in appearance with unenveloped nucleoprotein strands of myxo or oncornaviruses were observed with Dr. F. GYÖRKEY in most sarcoma cultures. In one instance, lymphocytes were grown from an angiosarcoma; these cells contained herpes-type virions. (vi) Bioassays. Human sarcoma cells are capable of altering the number and appearance of spleen foci, *i.e.* malignant transformed cell colonies, induced by the spleen focus forming Rauscher mouse leukaemia virus. When augmentation by human sarcoma cells of spleen foci occurs, a spleen focus-forming virus as measured by virus neutralization using mouse and human sera (Dr. F. GONZALEZ and Miss S. SCHNEIDER): mouse immune sera neutralize readily the mouse spleen focus-forming virus, whereas human sera from sarcoma-bearing patients neutralize the "recombinant" virus but not the mouse virus.

USE OF MINIATURE SWINE IN
VETERINARY MICROBIOLOGY AND IMMUNOLOGY,
ESPECIALLY FOR STUDYING SWINE ERYSIPELAS

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Göttingen miniature swine (MS) were used. They were sensitive to *Erysipelothrix insidiosa* and responded well to stimulating infection carried out by the Fortner-Dinter method. Skin and temperature reactions were more constant and uniform in MS than in breeding swine. The standard course of erysipelas manifested itself in this swine still more clearly than in the white mouse. The safety test of the inactivated adsorbate vaccine showed no fluctuations and could be evaluated in a reliable way. The potency tests of this vaccine proved also suitable in MS, since it was possible to make use of a sufficient number of animals for quantitative evaluation. MS were less susceptible to seasonal effects and to latent infections than breeding swine and white mice. The superiority of MS as a laboratory animal for the study of erysipelas in swine was especially clear in safety and potency tests of live erysipelas vaccine. These tests could be evaluated in a reliable manner. Breeding swine are less suitable, since individual animals respond in a different way and cannot be used in sufficient numbers. In white mice the course of safety and potency tests was so irregular that the results could not be evaluated. In preliminary experiments, MS appeared to be suitable for safety and potency tests of swine fever vaccines.

Bacteriology

MODEL OF THE CELL CYCLE OF *ESCHERICHIA COLI*

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The cell cycle of *Escherichia coli* T⁻ as revealed by phase contrast microscopy of single cells growing in slide cultures has been studied and a model has been constructed. Its essential features are: asymmetric unidirectional elongation of newborn cells; uniform speed of elongation during two thirds of the cell cycle; sudden doubling of the speed of elongation at intervals corresponding to the generation time even if cellular division occurs; increase in the rate of elongation at intervals corresponding to the generation time even if cellular division is inhibited by semisynthetic penicillins and filaments are formed.

EFFECT OF MEPACRINE UPON β -GALACTOSIDASE SYNTHESIS
IN *ESCHERICHIA COLI*

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Mepacrine has been proposed for fluorescent staining of chromosomes. WEISSBLUM and DEHASETH have recently shown that the dye had a specific affinity for deoxyadenylate-deoxythymidylate rich regions in DNA. Mepacrine-HCl was added at various concentrations to TMG-induced wild type *Escherichia coli* K-12 *lac*⁺ cells. The dye inhibited the synthesis of induced β -galactosidase and the incorporation of [¹⁴C]uracil and [¹⁴C]L-leucine. This inhibition was concentration dependent. Low doses (3–6 μ g/ml) of mepacrine-HCl did not decrease the radioactive uracil and L-leucine incorporation into acid-insoluble precipitate, but still inhibited beta-galactosidase formation. This fact allowed to assume that the promoter gene in the *lac* operon on which the RNA polymerase can initiate the transcription process, is a deoxyadenylate-deoxythymidylate rich region. Here the megacrine exhibits its inhibitory effect at lower concentrations than in other regions of the *E. coli* DNA. The kinetics of the inhibition was reminiscent of that of glucose repression, which could mean the inhibition of the initiation of mRNA synthesis. Similar results were obtained by examining inductive tryptophanase synthesis, which is also sensitive to catabolite repression. Preliminary observations with a catabolite insensitive *lac* promoter mutant strain showed no increased sensitivity to mepacrine HCl. This further supported the above hypothesis concerning the mode of action of mepacrine.

SERINE STARVATION AND RNA SYNTHESIS IN *ESCHERICHIA COLI*

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The exceptional effect of serine starvation on [¹⁴C] uracil incorporation has been studied in an isogenic *rel*⁺-*rel*⁻ mutant pair of *Escherichia coli* multi-auxotrophic for histidine, arginine and serine. In both strains, irrespective of their RNA synthesis control, about ten times as much uracil was incorporated during histidine starvation than during serine deprivation. This tenfold difference was decreased to threefold one on the simultaneous addition of all five purine-pyrimidine bases and the 18 amino acids. In cells treated with folate cycle inhibitors (trimethoprim or hydroxylamine) in the presence of the above-mentioned compounds, uracil incorporation decreased during histidine starva-

tion, but not during serine deprivation. The results indicate that (i) the stringent-relaxed regulation of RNA synthesis is not altered by serine starvation; (ii) the consequences of serine deprivation are not restricted to a single system; (iii) the severe shortage of relaxed RNA synthesis during serine starvation is a result of the inhibition of biosynthesis of (a) RNA building blocks, (b) many amino acids, and (c) a block of the dihydrofolate-tetrahydrofolate cycle.

NEOMYCIN, KANAMYCIN AND GENTAMICIN SENSITIVITY OF A DRUG-DEPENDENT *ESCHERICHIA COLI*

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A drug-dependent *Escherichia coli* B strain (which grows in streptomycin, paromomycin or ethanol) was found to be resistant to kanamycin in the presence of paromomycin, but streptomycin induced kanamycin sensitivity. The independent revertant mutant of GORINI was kanamycin resistant, whereas the authors' *E. coli* Sd-4-73 Drug^D strain and its independent SM^RPM^S revertant exhibited sensitivity to kanamycin A, neomycin B and gentamicin C in the absence of the required drugs on minimal or complete media. The sensitivity could not significantly be influenced by 10–1000 µg/ml streptomycin, but with paromomycin a considerable protection was attained against all three aminoglycosides in respect of their bacterostatic and bactericidal effect. In the case of other types of antibiotics, the quality and quantity of drug providing growth were indifferent. It is supposed that the interference of toxic aminoglycosides and paromomycin occurs on the surface of P10 protein of the 30S subunit. The neutrality of streptomycin can be explained by the fact that it is bound to a single site of the P10 protein, while paromomycin has many binding sites.

INDUCTION OF A LYTIC ENZYME IN *BACILLUS CEREUS* SPORES

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Induction of the lytic enzyme of *Bacillus cereus* 569 — supposed to be bound to episome — has been investigated. Induction is known to be brought about by the inhibition of DNA synthesis in vegetative cells. In order to study whether DNA synthesis was a precondition of induction spores of *B. cereus* were induced for lytic enzyme production. UV irradiation failed to exert any inductive action either on resting, or on activated or germinated spores.

However, a sensitivity to UV irradiation persisted under these conditions as indicated by the decreasing number of surviving spores after increased UV doses. In the stage of outgrowth the correlation between biosynthesis of macromolecules and enzyme induction has also been investigated. A thymine deficient mutant of *B. cereus* was isolated and spores of this mutant were cultured in a thymine-free medium. Starvation for thymine during outgrowth led to a massive induction of the lytic enzyme. This induction was not prevented by the inhibition of protein synthesis (with chloramphenicol). The inhibition of RNA synthesis by actinomycin and rifampicin elicited lysis of the culture, so no correlation between induction and RNA synthesis could be established.

DEGRADATION AND SYNTHESIS OF DEOXYRIBONUCLEIC ACID
IN ULTRAVIOLET IRRADIATED BACILLUS CEREUS CULTURES

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Degradation and synthesis of DNA were studied in induced cultures. DNA synthesis was followed by [³H] thymine incorporation. [³H] thymine was incorporated into a wild-type *B. cereus* 569 grown on 2.5 mg/ml casamine medium. Incorporation of [³H]thymine was less in UV treated cultures than in the control ones. The rate of DNA synthesis was measured with [³H]thymine pulse label. The irradiated cultures were shown to synthesize their DNA at a lower rate. DNA degradation was followed by measuring the acid-precipitable radioactivity of the [³H]thymine-labelled bacterial culture. Inductive and still larger doses produced an increase in the acid-soluble fraction. At 0 min after irradiation the inhibition of DNA synthesis, and thereafter a degradation of DNA, were observed. In connection with DNA degradation the nuclease activity of the irradiated culture was studied on DNA ³²P substrate. The enzyme extract prepared from the irradiated culture showed an about twofold DNase activity as compared with the control one. The role of ATP-dependent nuclease in lytic enzyme induction and in DNA degradation has also been investigated. The optimum ATP concentration of the ATP-dependent nuclease has been determined. At optimum concentration of 3×10^{-4} M ATP, DNase activity was fivefold of the original level. It was also observed that an acid soluble substance, extracted from the UV irradiated culture, exerted an inhibitory effect on DNase.

FACTORS INFLUENCING THE MULTIPLICATION OF THE ADENINE-DEPENDENT MUTANT OF *BACILLUS ANTHRACIS*

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In the lyase-deficient mutant of *Bacillus anthracis*, alkaline phosphatase synthesis proved to be derepressed. Production of the derepressed enzyme was accompanied by cell wall damages. Alkaline phosphatase synthesis of the mutant was derepressed by adenine, adenosine or adenine nucleotides. Growth of the mutant, even in the presence of high concentrations of adenine, never reached the growth rate of the prototrophic strain and similarly, ATP synthesis was less intensive in it than in the prototroph. Raising of the adenine concentration beyond certain level inhibited the mutant's growth rate. Among adenine, adenosine, deoxyadenosine and adenine nucleotides, adenine induced the most intensive growth of the auxotroph. With adenosine at the same concentration, growth was considerably less intensive. The mutant's sonicated cell suspension elicited adenosine deaminase activity. Experiments with [¹⁴C]adenine showed that adenosine deaminase participates indirectly in the adenine metabolism of the mutant. The growth of the mutant in the presence of ¹⁴C stops at a low level even in a considerable amount of adenine is still present in the medium. Part of the [¹⁴C]adenine was incorporated and the rest transformed into inosine or hypoxanthine. For the loss of virulence the slow growth of the adenine-deficient mutant may be responsible.

LIFE CYCLE OF *STREPTOMYCES GRISEUS* 45-H/G-20

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As a model of differentiation, the life cycle of *Streptomyces griseus* 45-H/G-20 was examined. The changes of enzyme activities, biochemical markers and resistance to ultrasonication of mycelial elements were examined during cultivation. Five stages of growth were distinguished on the basis of cytomorphological traits and quantitative changes of the markers: germination phase, exponential growth phase, lytic phase, secondary growth phase and stationary growth phase. The germination phase is characterized by a decrease in resistance to ultrasonication; the exponential growth phase by the peaks of β -galactosidase and β -glucosidase activities; the lytic phase, by the first peaks of alkaline phosphatase and alkaline protease; the secondary growth phase, by the maximum of factor C in the fermentation liquid and by the appearance of elements resistant to ultrasonication; the second peaks of

alkaline phosphatase and alkaline protease the peak of melanin concentration and the maximum number of spores resistant to ultrasonication were found in the stationary growth phase. The markers examined and the cytomorphological changes during development contribute to a better understanding of the differentiation process.

LYSOGENIC *BACILLUS CEREUS* STRAINS

ERZSÉBET NAGY and VERA GAÁL

Institute of Microbiology, University Medical School, Szeged

After the addition of 0.5 $\mu\text{g/ml}$ mitomycin C to exponentially growing liquid cultures of 10 *Bacillus cereus* strains, a short period of residual growth followed by rapid lysis was observed. Studying the lysates of these strains, attempts were made to detect phage effects on several *B. cereus*, *B. anthracis* and *B. megaterium* strains as indicators by using the plaque method. Since by this method indicator strains sensitive to the presumed phages were found only for two strains, the lysates were concentrated and purified by cyclic centrifugation, and negatively stained concentrates were examined by electron microscopy. It was found that as a result of mitomycin C induction, intact phage particles different in size and morphology were released from each of the 10 strains. In each of 3 strains, particles of two different types were demonstrated simultaneously. By means of acridine orange extinction, "cured" derivatives of the original strains could be produced which proved sensitive indicator strains for further phages.

COMPARISON OF FOOD POISONING *STAPHYLOCOCCUS AUREUS* STRAINS ON BAIRD-PARKER AND GIOLITTI-CANTONI MEDIA

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Central Veterinary Meat Control Laboratory, Budapest

Food samples were examined for *Staphylococcus aureus* on Baird-Parker (B-P) agar and Giolitti-Cantoni (G-C) media. Soft cheese was contaminated under laboratory conditions with 100, 1000 and 10 000 germs/g of the standard strain Jordan. To determine the most probable number and the actual count, G-C enrichment medium containing 0.025% agar and B-P egg-yolk agar medium, respectively, were used. The actual count could be determined in 48 hr, whereas the enrichment medium yielded results only after 72 hr, because a subculture had to be made on solid medium, preferably on blood agar.

STAPHYLOCOCCUS AUREUS L-FORM-INHIBITING SUBSTANCE
PRODUCED BY STAPHYLOCOCCUS AUREUS NY-23

M. FODOR

County Hospital, Nyíregyháza

It has been shown earlier that 40–50% of *Staphylococcus aureus* strains inhibited the growth of *S. aureus* L-forms. From the supernatant of the L-form-inhibiting strain Ny-23, a substance of non-protein character has been isolated which even in high dilutions inhibited *S. aureus* L-forms. The substance is produced in the presence of glucose or sucrose under aerobic conditions, with a pH optimum between 7 and 8. The inhibitor, which is inactivated at 90°C within 20 min, precipitates from the supernatant partially and completely at 30% and 50% ammonium sulphate saturation, respectively. The substance purified on molselect G 25, or DEAE Sephadex columns or by ultrafiltration has a molecular weight over 30 000. Its haemolytic activity on sheep, rabbit and human erythrocytes is weak; it intensely lyses human leucocytes. The latter effect is completely suspended with pooled human serum inactivated at 56°C without affecting the L-form-inhibiting capacity of the substance. The absorption spectrum of the purified substance in 0.1 N NaOH has peaks at 217 and 230 nm.

ANTIBIOTIC RESISTANCE OF STAPHYLOCOCCUS AUREUS
HOSPITAL STRAINS ISOLATED IN THE YEARS 1958–1972, IN THE LIGHT
OF ANTIBIOTIC CONSUMPTION IN HUNGARY

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*László Hospital for Infectious Diseases, Budapest and National Institute
of Public Health, Budapest*

The resistance to penicillin, streptomycin, tetracycline, chloramphenicol and erythromycin of *Staphylococcus aureus* strains isolated from routine material was followed up and plotted against the consumption of antibiotics in Hungary. The antibiotic resistance to each of the above antibiotics ran roughly parallel in the period 1961 to 1969. There was a definite correlation between the development of penicillin-resistance and of multiple resistance. Initially, there was a gradual increase in the resistance of the isolates, culminating between 1963 and 1966 and declining thereafter. While the tetracyclines are believed to be responsible for the selection of multiple-resistant strains, the decline in resistance may be attributed first of all to penicillinase-resistant penicillins.

DETECTION BY PASSIVE HAEMAGGLUTINATION OF *PSEUDOMONAS AERUGINOSA* ANTIBODIES IN PATIENTS SERA

MARIA M. ÁDÁM, B. LÁNYI and G. PETRÁS

National Institute of Public Health, Budapest

A total of 146 serum specimens from 24 patients admitted to an acute respiratory ward was examined. From 17 patients *Pseudomonas aeruginosa* strains belonging to LÁNYI's serogroups O1, O2, O3a,3d, O3d,3f, O4a,4b, O4a,4d, O5a,5d, O6, O7a,7b, O7a,7c, O8, O10a, O11 and O13 were cultured. The same patient frequently yielded 2 to 8 different serogroups. Serogroups colonizing the patients for prolonged periods induced a 4 to 64-fold increase in the corresponding antibody titre. After eliminating the infection, the titres fell to the initial level in 3 to 6 weeks.

BACTERIOCIN-TYPING

K. CSISZÁR

Public Health Station, Salgótarján

Escherichia coli associated with infantile enteritis, serologically untyped *E. coli*, as well as *Shigella* and *Salmonella* strains, all isolated from routine material, have been tested for colicinogeny and sensitivity to standard colicinogenic strains. *Pseudomonas aeruginosa* strains have been typed on the basis of pyocinogeny.

DIFFERENTIATION OF *HAEMOPHILUS* SPECIES

ZSUZSANNA CSUKÁS

Institute of Microbiology, Semmelweis University Medical School, Budapest

One-hundred fifty *Haemophilus* strains isolated from man have been identified on the basis of their growth factor requirements and biochemical characteristics. The growth factor-determining methods of ALEXANDER, COWAN-STEEL and PICKETT-STEWART were compared. The best reproducibility and results agreeing best with the biochemical features were obtained with ALEXANDER's method. Ten per cent of the cultures could not be identified by PICKETT and STEWART's technique. Of the biochemical methods, sucrose and xylose fermentation and indole production proved to be the most useful for species differentiation. *H. influenzae* strains did not ferment sucrose and their majority was xylose and indole positive. *H. parainfluenzae* and *H. parahaemolyticus* strains fermented sucrose and were xylose and indole negative.

SEROTYPES OF ATYPICAL MYCOBACTERIA

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Atypical mycobacteria mainly of the *Mycobacterium avium* group isolated from various animals were investigated. Twenty strains were obtained from different varieties of poultry and birds in the zoo, 10 from cattle, 20 from swine, and 10 from miscellaneous materials. SCHAEFER's method was used for serotyping. Twenty-five and 12 strains belonged to serotype II and I, respectively, the remainder to other serotypes. The results may be helpful in solving epidemiological problems concerning *M. avium* infections.

BIOLOGICAL CHARACTERIZATION OF LEPTOSPIRA CANICOLA STRAINS
ISOLATED IN HUNGARY

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The increasing incidence of human canicola fever in Hungary has called attention to canine leptospirosis. In the last two years, 284 dogs were examined serologically for leptospirosis; 35 proved positive for *Leptospira canicola*. From the kidneys and urine samples of these dogs, *L. canicola* was isolated by culturing or in experimental animals. The ground-squirrel, the chinchilla and the Syrian hamster proved to be most sensitive; guinea pigs usually survived the infection. The strains grew well in Korthof medium, produced haemolysin and lipase. Of the 7 strains, 3 lost their virulence during one-year culturing. In contrast to observations published by others, rats and mice were successfully infected with *L. canicola*, but after 2-5 weeks no leptospire could be reisolated from the kidneys of these animals. *L. canicola* infection induced immunity to *L. icterohaemorrhagiae* and *L. pomona*, but there was no cross immunity with the lipase-producing species. Selection of strains of good antigenicity allowed a successful immunization of dogs against canicola leptospirosis. The importance of vaccination is discussed.

BIOASSAY OF ESCHERICHIA COLI ENTEROTOXIN

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The purpose of this work was to study the effects of the heat stable enterotoxin obtained from enterotoxic *Escherichia coli* and of congener sub-

stances (enterotoxin of *Vibrio cholerae*; *E. coli* endotoxin) on vascular and intestinal smooth muscle. Specific aims were to contribute to the knowledge of the mechanism of action and to find a bioassay test for these agents. Vascular smooth muscle preparations, and relaxant effect of enterotoxin on noradrenaline contraction in rabbit aorta, offered reproducible results and constituted the simplest reliable method of testing. *V. cholerae* enterotoxin (FINKELSTEIN or BURROWS' method) and *E. coli* endotoxin had no effect on the noradrenaline contracted rabbit aorta. A variety of models were suggestive of enterotoxin interference with exogenous and perhaps endogenous catecholamines. (i) Enterotoxin appeared to cause on smooth muscle effects which were opposite to those of adrenergic activators. (ii) In its interference with noradrenaline, enterotoxin mimicked the effects of phentolamine on the rabbit aorta. (iii) The ability of enterotoxin to neutralize the effect of isoproterenol on the dog jejunal villi, suggested that through the implicated receptor sites enterotoxin is capable of specifically neutralizing beta type catecholamine activation. Such actions may play a decisive role in enteric disease. The mechanism of action of *E. coli* enterotoxin has been discussed in the light of the findings.

PARALLEL BACTERIOLOGICAL AND SEROLOGICAL TESTING OF CHILDREN WITH URINARY ESCHERICHIA COLI INFECTION

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Using passive haemagglutination method the diagnostic value of *Escherichia coli* antibody titres, and their changes in time were evaluated in children with urinary infections. In the haemagglutination tests, homologous bacterial strains were used. A titre of 1 : 64 was regarded as normal. Besides serotyping, the haemolysin-producing capacity of *E. coli* strains cultured from the urine of 34 patients with a count over 10^5 per ml, was determined. Increased antibody titres were found in all patients with pyelonephritis, whereas only in a single case of cystitis. Changes in time of antibody titres differed in acute and in chronic pyelonephritis. Seventeen of the *E. coli* strains could be serotyped and they fell into 8 serological groups; antigens O4 and L6 being the most frequent ones. No correlation was found between serological groups and clinical diagnosis.

MYCOBACTERIOCIINOGENIC MYCOBACTERIUM RANAE STRAINS

I. SZABÓ

Korányi Institute for Tuberculosis and Pulmonology, Budapest

Four strains have been isolated which agreed in all the examined characteristics with *Mycobacterium ranae* except that they slightly decomposed one or two amides besides amides 1, 3, 5, 6 and 8 of the BÖNICKE series. Two of the strains have been re-examined by BÖNICKE and WAYNE. In their opinion, too, the strains were not identical with *M. ranae*. They were resistant to the homologous phages. Furthermore, addition of the strains to a phage-sensitive homologous strain resulted in lysis of the latter. This was due to bacteriocinogenesis, not to lysogenesis. In their pathogenicity for animals, the bacteriocinogenic strains did not differ from the non-bacteriocinogenic strains.

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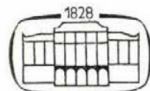
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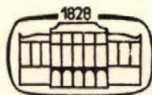
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COMBINED PHYTOHAEMAGGLUTININ AND DIANHYDRODULCITOL TREATMENT OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS INFECTION IN MICE

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Summary. The course of intracerebral lymphocytic choriomeningitis (LCM) virus infection was studied in mice treated simultaneously with dianhydrodulcitol (DAD) and phytohaemagglutinin (PHA). Earlier experiments revealed that DAD decreased and PHA enhanced the cellular immune response of mice to LCM virus infection. On applying the treatments simultaneously they inhibited each other and neither the decreasing effect of DAD nor the enhancing effect of PHA on the cellular immune response could be observed.

It has been shown earlier that both phytohaemagglutinin (PHA) and dianhydrodulcitol (DAD) influenced the course of intracerebral lymphocytic choriomeningitis (LCM) virus infection in mice. Characteristic lymphocytic choriomeningitis developed earlier in mice infected with 10^{-1} – 10^{-3} virus dilutions and the mortality rate was higher using 10^{-4} – 10^{-5} virus dilutions if the mice were given 10 mg/kg of purified phytohaemagglutinin than in animals left untreated. The results indicated that PHA enhanced the cellular immune response to LCM virus infection of mice [1]. Using DAD treatment and LCM virus infection, death ensued later and choriomeningitis failed to develop in 50% of animals, part of which became virus carrier. The results indicated that the cytostatic agent and LCM virus influenced each other's effect. While DAD reduced the cellular immune reaction to LCM virus infection, the persisting virus aggravated the lymphoid atrophy caused by the cytostatic agent [2]. In the present experiments, the course of LCM virus infection was studied in mice subjected to simultaneous PHA and DAD treatment.

Materials and methods

Experimental animals. Six-week old Swiss mice were used in groups of 20.

Dianhydrodulcitol treatment. DAD substance (Chinoin Pharmaceutical and Chemical Works, Budapest) was dissolved in distilled water. In case of a single intravenous injection the LD₅₀/21 days was 15 mg/kg for the mice.

Phytohaemagglutinin treatment. Purified phytohaemagglutinin (Lot Br7 Wellcome Research Laboratories) was used. In the case of a single intravenous injection the LD₅₀ was 20 mg/kg.

LCM virus infection. The W.E. strain used in the experiment was maintained through serial mouse passages in our institute. Mice were given a dose of 100 LD₅₀ intracerebrally of the pretitrated virus.

Virus reisolation. Mouse-groups were infected intracerebrally with blood samples obtained by orbital puncture and with 1:10 dilution of brain suspension of animals surviving the virus infection. The presence of LCM virus was indicated by characteristic neurological symptoms.

Study of the lymphoid system. The relative spleen weight of succumbed animals and of those sacrificed 11 days following LCM virus infection was determined. Relative spleen

$$\text{weight} = \frac{\text{spleen weight, mg}}{\text{body weight, g.}}$$

Results

Treatment applied in the individual experimental groups is presented in Table I. DAD treatment with a dose of 0.10 LD₅₀ was started 11 days before LCM virus infection and applied every second day before and every day after infection. One day before infection the animals were given 10 mg/kg

Table I
Experimental mouse groups

Group	PHA	DAD	LCM	PHA-LCM	DAD-LCM	DAD-PHA LCM	Control
Intravenous treatment	PHA	S	S	PHA	S	PHA	S
Intracerebral infection	S	S	LCM	LCM	LCM	LCM	S
Intraperitoneal treatment	S	DAD	S	S	DAD	DAD	S

PHA = phytohaemagglutinin

DAD = dianhydrotolucitol

LCM = lymphocytic choriomeningitis virus

S = physiological saline

of PHA. Typical neurological symptoms (trembling, seizures) and death were recorded. Animals surviving the LCM virus infection were sacrificed on the 21st day after infection. During the experiment no death occurred. The time of death in the LCM virus infected groups is presented in Fig. 1.

All mice of the LCM group succumbed on the 7th–10th day, displaying symptoms typical of lymphocytic choriomeningitis. Survival was longer in the DAD-LCM group and only 40% of the animals showed symptoms of choriomeningitis. These animals died on the 7th–10th day. Ten per cent were alive on the 21st day, and became virus carriers as proved by reisolations.

In the PHA-LCM group, the neurological symptoms and death occurred earlier than in the LCM group; 100% of the animals succumbed on the 6th–

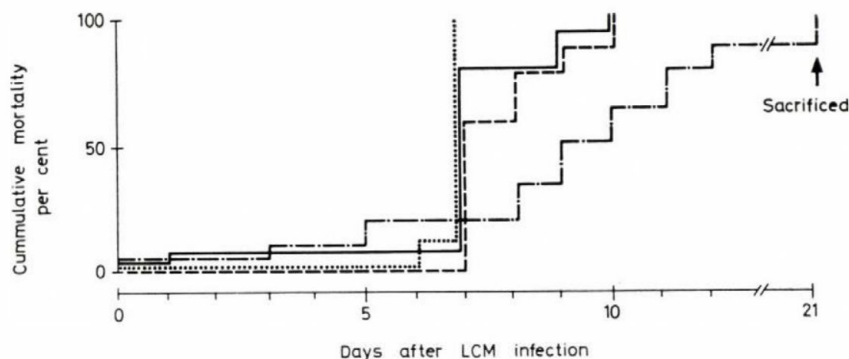


Fig. 1. Death of LCM virus-infected animals after dianhydrolulcitol and phytohaemagglutinin treatment

7th day. In the DAD-PHA-LCM group the time of appearance of neurological symptoms as well as the time of death were practically identical as in the LCM group, and all mice succumbed on the 7th–10th day. Relative spleen weight was determined on the 7th–10th day after LCM virus infection. It was 4.9 in the control group, while 4.2, 3.4 and 3.1 in the LCM, DAD and DAD-LCM groups, respectively. In the PHA and PHA-LCM groups, values of 6.0 and 5.3 were obtained. In the DAD-PHA-LCM group the mean was 4.4.

Discussion

The basis of lymphocytic choriomeningitis caused by intracerebral LCM virus infection is a cellular immune response, similarly as in the homograft reaction [3].

Our own experiments as well as the results of other investigators have shown that treatments causing an attenuation of the immune response, inhibit the development of the characteristic symptoms and histological changes of lymphocytic choriomeningitis, and part of the animals becomes virus carrier [4–19]. This was the case in our earlier experiments in DAD-treated and LCM virus-infected animals [2] as well as in the DAD-LCM group in the present experiments.

Findings concerning the influence on the cellular immune response of phytohaemagglutinin are contradictory. The effect depends on the mitogenic activity of the preparation, on its dose and the way of application [20, 21].

A single intravenous injection of purified phytohaemagglutinin was found to enhance the immune response to LCM infection in both our earlier [1] and the present investigations. Mice of the PHA-LCM group died earlier than those subjected only to virus infection. The combination of DAD and

PHA failed to exert either a reducing or an enhancing effect on the cellular immune response in mice of the DAD-PHA-LCM group, thus they counteracted or prevented each other's effect. The agents used in our earlier experiments had opposite effects on the lymphoid system: DAD caused atrophy and PHA hypertrophy in the spleen [1, 2]. These effects were observed in the present experiments, too. LCM virus infection is known to cause lymphoid atrophy in mice [22]. In agreement with this observation, relative spleen weight was lowest in the DAD-LCM group as a consequence of the atrophy caused by both treatments. Values for the PHA-LCM group were only slightly above the control, indicating that the atrophy due to LCM virus and the hypertrophy due to PHA had interfered with each other. The same occurred in the DAD-PHA-LCM group. A direct correlation was found between the relative spleen weight and the decreased cellular immune response. Under the effect of combined treatment, mean spleen weight was comparable to the control value and the cellular immune response was also normal. In the clinical respect we consider it significant, that a single intravenous dose of PHA prevented the immune response-decreasing effect of the simultaneously applied cytostatic agent.

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PATHOGENICITY OF AVIAN MYCOPLASMA SEROTYPES FOR EMBRYONATED CHICKEN EGGS*

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Summary. Forty-seven strains of avian mycoplasma, representing 8 serotypes (A, B, C, E, L, M, P and R) were tested for pathogenicity to embryonated chicken eggs. Serotypes A and R were distinctly pathogenic, serotype C was slightly pathogenic whereas serotypes B, E, L, M and P were non-pathogenic.

On the basis of biochemical characteristics, pathogenic species of avian mycoplasma have been distinguished [1]. Since avirulent strains may occur amongst the former, it would be difficult to consider one species pathogenic and another non-pathogenic unless the pathogenicity of biochemically identified "pathogenic" strains for embryonated chicken eggs and for adult birds was tested. It should be noted that, in addition to *Mycoplasma gallisepticum* (serotype A), other species or serotypes, too are potential pathogens [2–4].

Since in a previous study [5] we found that not only serotype A but also B, C, E, L, M, P, I and R are prevalent, it was interesting to investigate the pathogenicity of avian mycoplasma species belonging to different serotypes [6] for poultry birds and embryonated eggs *via* yolk sac inoculation.

Materials and methods

Eggs. Fresh hatching eggs were obtained from a flock having no history of mycoplasma infection. Before performing the pathogenicity tests, the eggs were treated with erythromycin [7] to eliminate mycoplasma infection by the trans-ovarian route. The eggs were washed with soap and water then with 70% alcohol. After drying and incubating at 37°C for 6 hr the eggs were immersed in chilled erythromycin solution (0.1%) at 4°C for 30 min, then dried at room temperature and incubated at 37°C for 7 days. On the 8th day the eggs were checked by candling.

Mycoplasma strains. Forty-seven avian mycoplasma strains belonging to 8 serotypes were used. The number of strains according to serotypes was: A, 25; B, 2; C, 2; E, 2; L, 5; M, 3; P, 1; R, 7.

Inoculation of embryonated chicken eggs. Before carrying out pathogenicity studies, the mycoplasma strains were passaged three times in 7-day embryonated eggs so as to regain

* Part of a Ph. D. Thesis submitted to the Postgraduate Institute of Medical Education and Research, Chandigarh, India.

virulence in the natural host; 0.2 ml aliquots of 24 hr broth cultures were inoculated into the yolk sac. Incubation lasted at 37°C for a week. The yolk was harvested at the third passage for pathogenicity test. As control, plain PPLO broth was passaged three times. Simultaneously, the yolk of uninoculated eggs was collected and passaged three times to check for the presence of natural mycoplasma infection of the eggs.

Two hundred eighty five embryonated eggs, including 50 controls, were inoculated. Each test strain was inoculated in 5 eggs. Incubation at 37°C lasted for 12 days with daily candling. Embryos dying within 48 hr after inoculation were discarded. Dead embryos were examined for gross lesions. The yolk and specimens from the lesions were collected and cultured for mycoplasma. Each strain isolated in this manner was tested for identity with the inoculated test strain.

Results

Pathogenic and non-pathogenic properties were considered on the basis of embryo mortality. None of the strains of serotypes B, E, G, L, M and P was pathogenic, since all embryos survived up to the termination of the experiment (12 days).

In serotype A, 2 out of 25 strains were non-pathogenic. The remaining 23 strains caused 75–100% mortality within 9 days (usually 3–6 days) after inoculation; these were, accordingly, considered pathogenic. One strain of serotype C and 4 strains of serotype R caused nearly 50% and more than 50% mortality within 3–7 days, respectively; thus the former was regarded slightly pathogenic, the latter pathogenic. One strain of serotype C and 3 strains of serotype R were non-pathogenic.

The embryos died of infection were considerably stunted and fragile as compared to the controls. There was an enlargement of the liver in some of them and others showed marked cutaneous haemorrhage and oedema. The caseous deposit from the abdominal cavity yielded mycoplasma species identical with the inoculated ones; none of the specimens contained bacteria and yolk from the controls was sterile.

Discussion

Two out of 25 strains of serotype A, including one strain recovered from a bird with chronic respiratory disease (probably infectious coryza), were pathogenic causing 75–100% embryo mortality. Strains of serotype R were partly pathogenic, partly apathogenic, whereas one strain of serotype C was slightly pathogenic. None of the strains of serotypes B, E, L, M and P caused fatal infection. Our findings regarding the pathogenicity of serotypes A, B, C, L, M and P agree with the observations of other authors [2, 3, 7]. Non-pathogenicity of some serotype A and R strains could be attributed to a complete loss of virulence for embryos or to an insufficient number of embryo passages to regain virulence.

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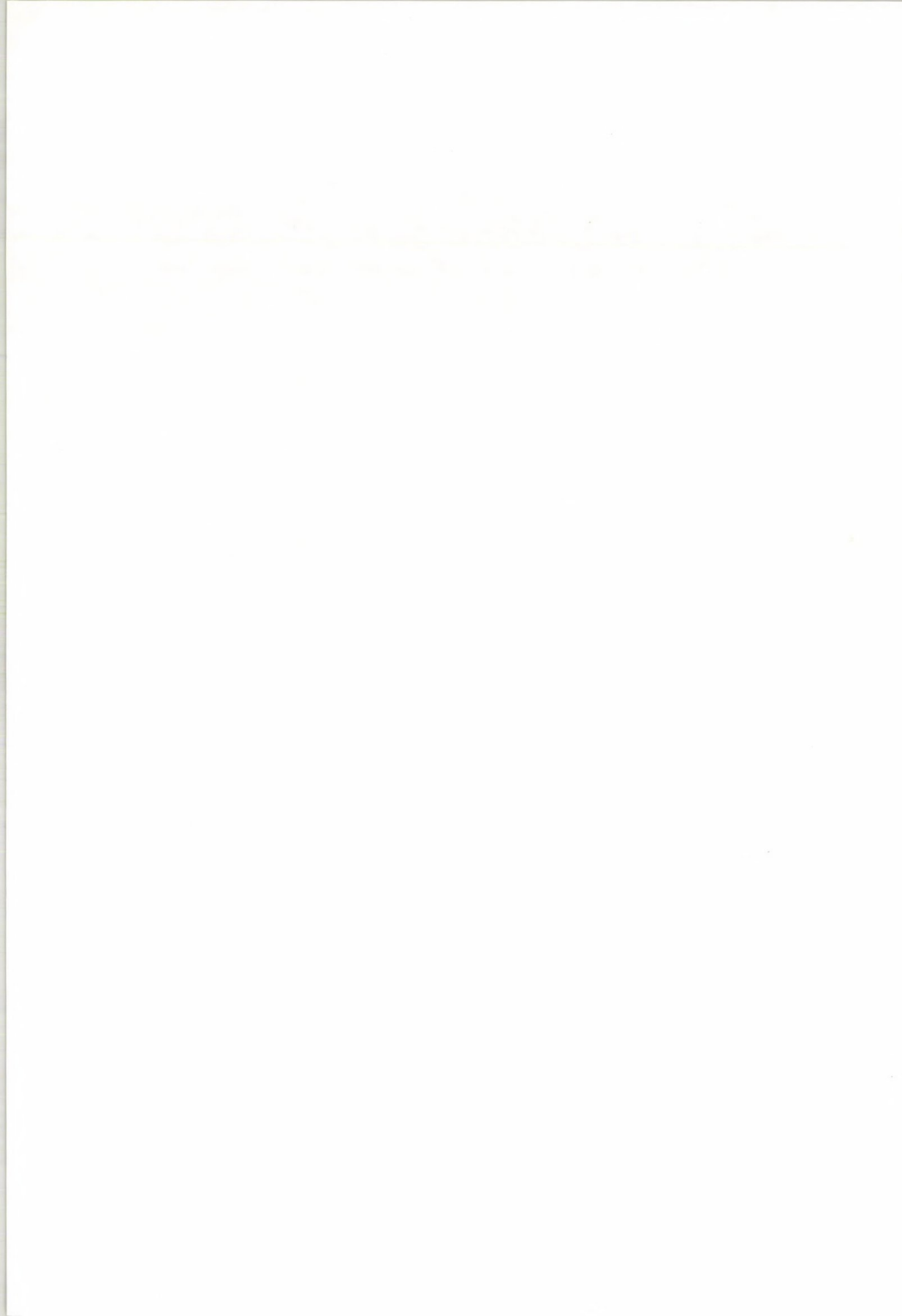
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EFFECT OF AUROTHIOGLUCOSE ON SOME ALLERGIC AND NON-ALLERGIC SKIN REACTIONS IN ANIMALS

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Summary. The antiphlogistic effect of aurothioglucose was examined in reversed passive Arthus reaction, delayed-type hypersensitivity and carrageenan oedema. A close association existed between the antiphlogistic effect of aurothioglucose and the pathogenetic role of polymorphonuclear leukocytes. In reversed passive Arthus reaction and in carrageenan oedema the drug decreased the inflammation considerably, whereas in delayed hypersensitivity, where the leukocytes play a less important part, it exerted only a mitigating effect.

In recent years several authors have reported on the effect of gold salts on lysosomes and lysosomal enzymes. PERSELLIN and ZIFF [1] showed *in vitro* that the gold salt accumulated in the lysosomal fraction and inhibited the activity of lysosomal enzymes. In the opinion of ENNIS *et al.* [2], colloidal gold preparations exert their therapeutic effect by inhibiting hydrolases. CHATEL *et al.* [3] described that prolonged gold treatment inhibited the activity of lysosomal enzymes in man. LÉVAI *et al.* [4] confirmed that gold salts accumulated in the lysosome. HERMAN *et al.* [5] have shown that in the central nervous system (hypothalamus) aurothioglucose is mainly bound to the lysosome.

In our institute, SZILÁGYI *et al.* [6] demonstrated that local treatment with gold salt inhibited the development of local Shwartzman reaction.

This paper presents data for the mechanisms of local gold salt treatment in allergic and non-allergic inflammatory reactions. As an early type reaction, the reversed passive Arthus reaction was chosen; delayed type reaction was elicited by ovalbumin + Freund adjuvant. Non-allergic inflammation was induced with carrageenan.

Materials and methods

Reversed passive Arthus reaction was produced in white female guinea pigs weighing 300 g. The animals were injected intravenously with 2 mg of ovalbumin dissolved in saline. After 15 min, 200 μ g antiovalbumin rabbit serum + 0.1 ml 20% aurothioglucose solution (Solganal B oleosum, Schering) were injected intracutaneously at several sites of the shaven dorsal skin. Control injections into different sites consisted of 200 μ g antiovalbumin serum +

the supernatant of the centrifuged aurothioglucose solution. Size and intensity of inflammation were read after 2 and 4 hr.

Delayed-type allergic skin reaction was produced by injecting 4 μ g ovalbumin dissolved in complete Freund adjuvant into the connective tissue of the foot-pad of female white guinea pigs weighing 300 g. After 8 days, 6 μ g ovalbumin + 0.1 ml 20% aurothioglucose were injected intracutaneously into the shaven dorsal skin. Control injections consisted of ovalbumin + gold-free solvent. The animals were sacrificed after 24 hr and the reaction was read after 24 hr by inspecting the inner surface of the skin.

Carrageenan oedema was elicited by injecting 0.1 ml carrageenan solution (1% in saline) into the foot-pad of female Wistar rats weighing 100 g, then by giving them 0.1 ml 20% aurothioglucose intracutaneously. Controls were injected with 0.1 ml amounts of gold-free solvent intracutaneously 24 hr before treatment. The reaction was evaluated after 3 and 24 hr.

Results

Results of reversed passive Arthus reaction are summarized in Table I. It was concluded that the intensity of the reaction considerably decreased under the effect of local gold treatment, and the inflammatory area was reduced to about half of the size of the control.

Table I
Inhibition by aurothioglucose of reversed passive Arthus reaction in guinea pigs

No. of animals	Mean size of reaction area, mm ²		Average intensity of reaction*	
	control	aurothioglucose	control	aurothioglucose
15	85 $\pm$ 10	43 $\pm$ 7	+++	+

* + weak, +++ marked

Delayed hypersensitivity elicited with ovalbumin + complete Freund adjuvant was also significantly decreased by aurothioglucose; the decrease concerned mainly the intensity of the reaction; its size was reduced less markedly (Table II).

Table II
Inhibition by aurothioglucose of delayed-type hypersensitivity in guinea pigs

No. of animals	Mean size of reaction area, mm ²		Average intensity of reaction*	
	control	aurothioglucose	control	aurothioglucose
15	53 $\pm$ 5	38 $\pm$ 5	+++	+

* = weak, +++ marked

Foot-pad oedema induced by carrageenan was checked at regular intervals. Three hours after the injection both gold-treated and control pads showed a similar degree of oedema. Some hours later the oedema disappeared on the treated pads; on the controls it remained marked even after 24 hr (Table III).

Table III

Effect of local aurothioglucose injection on carrageenan oedema in rats

Degree of foot-pad oedema*					
Control group			Treated group		
No. of animals	3 hr	24 hr	No. of animals	3 hr	24 hr
13	++++	++	15	++++	—

* — nil, ++ marked oedema, ++++ severe oedema

Discussion

In the early stage of reversed passive Arthus reaction histamine and serotonin are released, but for the full development of the reaction, lysosomal enzymes liberated from polymorphonuclear leukocytes are responsible [7]. HUMPHREY [8] showed that a reversed passive Arthus reaction could not be elicited in granulocytopenic animals and it was found [9] that no Arthus reaction developed in cooled animals. The inhibitory effect is mainly due to a hindering of the migration of polymorphonuclear leukocytes.

The present experiments have shown that aurothioglucose is inactive in the early phase of the Arthus reaction; its antiphlogistic effect in the leukocytic stage confirms the primary pathogenetic role of the lysosomal enzymes of polymorphonuclear leukocytes. Recent investigations [10, 11] indicate that the antiphlogistic effect of gold salts is based upon the inactivation of lysosomal enzymes.

In delayed-type allergic reactions polymorphonuclear leukocytes are present in addition to the predominating mononuclear cells [12]. The lysosomal enzymes probably play a role in initiating the reaction and in increasing the tissue lesions. WINTER *et al.* [13] were the first to describe that carrageenan, the polysaccharide of the alga *Chondrus crispus*, elicited oedema of the rat's foot-pad. It was assumed that the inflammation is started by histamine and bradykinin, but in later stages polymorphonuclear leukocytes play the main role. Our experiments have shown that gold salt is ineffective in the early stage of carrageenan oedema, but it exerts an antiphlogistic effect in the cellular phase.

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EFFECT OF HYPOTHERMIA AND ENDOTOXIN ON PHAGOCYTOSIS

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Summary. Experiments with colloidal ^{198}Au indicated that gold clearance highly decreased in rabbits cooled to 29–30°C. The capacity of storing colloidal gold decreased especially in the spleen and liver. Endotoxin reduced the gold clearance in normothermic rabbits; the decrease was more marked in hypothermic animals.

Hypothermia has been shown to inhibit the development of reversed passive Arthus reaction [1], in which the ingestion of the antigen-antibody complex by polymorphonuclear leukocytes plays an important role [2]. Our recent experiments have shown that the generalized Shwartzman reaction is inhibited in the hypothermic rabbit [3]. It is assumed that the generalized Shwartzman reaction is associated with the effect of endotoxin on the RES.

In the present work the effect of hypothermia and endotoxin on phagocytosis, and endotoxin-induced changes in the capacity of organs of storing colloidal gold were examined.

Materials and methods

Animals. Rabbits of both sexes weighing 2.5–3 kg were used. The animals were kept under standard conditions, fed a standard diet and allowed water *ad libitum*.

Colloidal ^{198}Au containing 2 mg gold per ml with an activity of 0.1 mCi/kg, was prepared by the Institute of Isotope Research, Hungarian Academy of Sciences, Budapest.

Hypothermia. The animals were covered with ice-water bags; to prevent excitation, thiobarbital (0.02 g/kg) was given to both the cooled and the control animals. Cooling to 29–30°C was checked by taking the rectal temperature.

Blood specimens. At 5, 15 and 28 min intervals after ^{198}Au injection, 0.1 ml samples of blood were drawn from the femoral vein into 0.2 ml of 3.3% sodium citrate.

Organ specimens. The animals were sacrificed between 30 and 40 min after ^{198}Au injection by bleeding through the carotid artery. One g samples from the liver, lung, spleen and kidney, and the whole left adrenal gland were examined. Activity was expressed as cpm/moist weight.

Radioactivity was measured in serological tubes in a Frieske-Hoepfner 49 apparatus equipped with a NaI(Tl) crystal scintillation detector.

Phagocytic index was calculated by using the equation

$$K = \frac{(\log C_0 - \log C_1)}{T_1 - T_0}$$

where C_1 and C_0 are the gold concentrations in the plasma at minutes T_1 and T_0 . Gold concentration in blood samples was expressed as cpm.

Escherichia coli endotoxin ($LD_{50} = 6$ mg) was prepared as described by BOIVIN *et al.* [4]. Twenty-four hours prior to gold clearance examination, the rabbits received intravenously an amount corresponding to LD_{20} .

Results

The phagocytic index in normothermic rabbits was 0.0625. As demonstrated in Fig. 1, in the cooled rabbits phagocytosis decreased considerably. In normothermic animals the spleen exhibited the highest phagocytic activity. Phagocytosis was of a lower degree in the liver and especially in the lungs, kidney and adrenal gland. Owing to the large volume of the liver, this

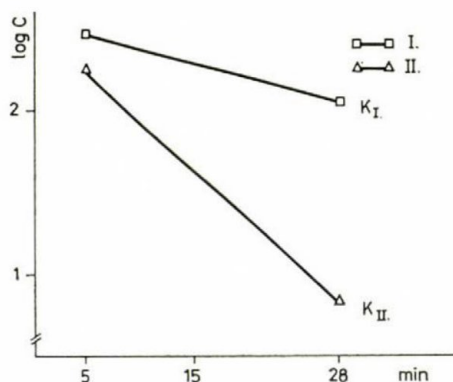


Fig. 1. Kinetics of the phagocytosis of colloidal ^{198}Au in rabbits; $\square$ — $\square$ hypothermic (29°C) animals, phagocytic index $K = 0.0187$; $\triangle$ — $\triangle$ normothermic (38°C) animals, phagocytic index $K = 0.0625$. Means of 3 experiments

organ contained the highest absolute amount of gold. In cooled animals gold concentration decreased approximately to 1/6 in the spleen and approximately to 1/2 in the liver. In the lung and adrenal gland there was no significant difference from the control; in the kidney the activity decreased (Fig. 2).

In normothermic animals, endotoxin decreased the stored amount of gold; in the hypothermic rabbits, it caused a further decrease of the phagocytic index (Fig. 3).

Discussion

Our data indicate that hypothermia hinders the uptake of ^{198}Au . The decreased activity in the spleen and liver are associated with the inhibition of RES in the whole body. Among the organs, only the hypothermic kidney

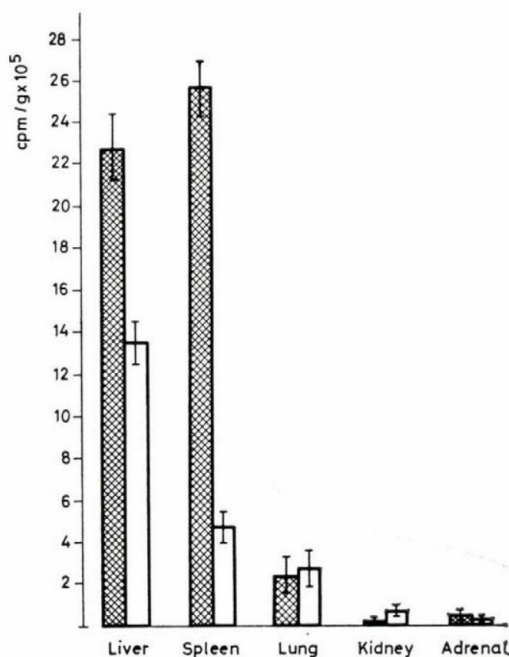


Fig. 2. Uptake of colloidal ^{198}Au by organs; hatched columns: normothermic (38°C) animals, clear columns: hypothermic (29°C) animals. Means of 3 experiments

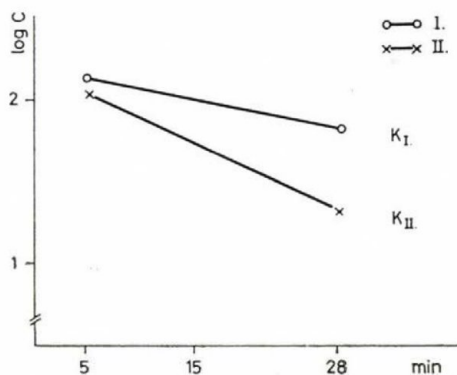


Fig. 3. Kinetics of the phagocytosis of colloidal ^{198}Au in rabbits after endotoxin injection; $\circ$ — $\circ$ hypothermic (29°C) animals, phagocytic index $K = 0.0164$; $\times$ — $\times$ normothermic (38°C) animals, phagocytic index $K = 0.0313$. Means of 3 experiments

contained more gold than the control, but in absolute amount the difference was slight. This finding may be explained less by an increased storage than by the fact that in the hypothermic animal tubular excretion and glomerular filtration decreases more considerably than does renal clearance [5]. These findings have confirmed that endotoxin injection results in a blocking of the RES, and that in cooled animals the blocking is even more marked.

It is known from the experiments of LUDÁNY *et al.* [6] that the phagocytic activity of polymorphonuclear leukocytes is an energy-consuming process accompanied by an increase in glucose utilization. SIESS [7] and GORDON and KING [8] showed *in vitro* that phagocytosis decreases significantly at low temperatures. According to BRAUER *et al.* [9] the degree of hypothermia is proportional to the decrease of RES activity.

Thus, the inhibitory action of hypothermia on reversed passive Arthus reaction should, in addition to other factors [10], be attributed to a decreased phagocytosis by polymorphonuclear leukocytes of the antigen-antibody complex.

As it is accepted that in generalized Shwartzman reaction the first endotoxin dose causes a blocking of the RES, it is improbable that hypothermia, which itself decreases RES activity, should inhibit the development of the reaction by influencing the RES.

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A NEW FUNGAL ISOLATE FROM PASPALUM SCROBICULATUM, LINN. WITH NEW BIOLOGICALLY ACTIVE METABOLITES

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Summary. A new fungal isolate from the host plant *Paspalum scrobiculatum* Linn., is reported. It is characterized and designated as a separate species *Phomopsis paspali* (n. sp.), not only for the different morphological characters as compared with the commonly occurring *Phomopsis* species in India, but also in view of its distinctive feature of producing biologically active new metabolites, cytochalasans. In addition, the genus *Phomopsis* has not been reported on this plant anywhere in the world.

Paspalum scrobiculatum Linn. natural order *Graminae*, is a sturdy millet, cultivated in poor soils in many provinces of India, in the areas of comparatively high rainfall. The grain which has been known since centuries to be toxic for animals and humans, is still used as an important food material by villagers after detoxicating it in their own way. The aboriginal tribes in the country even now use the plant for hunting wild animals by filling a carcass with the toxic material and placing it so that the wild animals would consume it [1].

Attention was drawn to this plant owing to its toxicity. ABDUL SATTAR [2] observed a considerable damage to this crop in Bihar by an attack of a smut caused by the fungus *Sorosporium paspali*, Macalp. BROWN and RANK [3] showed that the forage poisoning of cattle fed on *Paspalum dilatatum* Poir, was due to *Claviceps paspali* Stevans and Hall, a fungus that infects grass in South America. BROWN [4] studied the life of *Claviceps paspali* Stevans and Hall, and reported that *Fusarium heterosporium* Nees, and *Cladosporium* sp. also infected the plant. RAMKRISHNAN and SUNDARAN [5] reported the occurrence of *Claviceps paspali* on *P. scrobiculatum* in South India. Ergotoxin was absent in the sclerotia. Accidental deaths of animals have been reported due to consumption of infected *P. scrobiculatum* grain. BHIDE and AIMEN [6] observed the effect of the ethanol extract of *P. scrobiculatum* grains; these authors believed that the toxic principle was in the husk.

A survey of the crop of *P. scrobiculatum* was carried out in the Western coastal track of India, where most fields showed a large proportion of plants affected by fungi. Samples were therefore collected from the different regions and subjected to studies for the isolation of fungal strains.

Materials and methods

Isolation of the organism. One of the commonly occurring fungal strains isolated from samples of the grain has been studied in detail. The seed surface was sterilized with 2% mercuric chloride by immersing the seed for 30 s in the solution and washing it repeatedly with distilled water to eliminate saprophytic fungi and mercuric chloride, which could be checked by testing the washings with AgNO_3 solution. As a medium, 2% potato dextrose agar (PDA) was used; it was autoclaved, cooled and poured in sterile Petri dishes. The seeds were sown on this medium and incubated at 24–28°C. Most of the seeds showed the presence of fungal infection. A single microorganism was isolated and cultured by picking up individual colonies on separate Petri dishes in which they were allowed to sporulate. In two to three weeks, pycnidia were produced. The conidia, pycnidia and mycelia were studied morphologically and the fungus was characterized taxonomically.

Separation of compounds I and II. The identified fungus was cultivated in large conical flasks on 2% PDA. After a 15–20 days period of growth the entire culture including the solid medium was extracted with ethanol in a percolator for about 4 days. This cold extraction was followed by the evaporation of the solvent under a fan, without application of any heat. The dried residue was fractionated by using fat solvents such as chloroform or ether. The ether extract was subjected to thin-layer chromatography on silica gel plates with chloroform:methanol (95:5) as the solvent system. The chromatogram was developed by spraying with 1% vanillin 50% o-phosphoric acid. Two distinct non-fluorescent and three fluorescent spots were noted; fluorescent contaminations were neglected.

The two compounds were further separated by column chromatography. A crude ether extract (2 g) was dissolved in 10 ml of dichloromethane and loaded on a column of 30 mm in diameter and 730 mm in height containing 100 g of silica gel. Benzene was used as the first eluent and the column was operated under direct ultraviolet light. The benzene was changed for chloroform and methanol in combinations adjusted according to the movement of the material. Seven fractions could be collected in all. The fractions were again checked by TLC on silica gel and it was found that compound I was separated in the 4th and 5th fractions associated with green and violet fluorescent contaminations. The 6th and 7th fractions showed only compound II, contaminated with a compound of blue fluorescence. Fractions 4 and 5 contained pure compound I, and fractions 6 and 7, compound II. These compounds were further purified by dissolving the residue in di-chloromethane and crystallizing it in di-chloromethane/di-isopropyl ether. Compound I weighed 550 mg and Compound II 316 mg. The physical properties of the purified compounds were studied in UV spectra and microanalyses were made and the toxic properties were studied in mice and dogs.

Results

Characteristics and systematic position. The grain incubated on PDA at 24–28°C for 48 hr showed a fungal growth in the form of a small white fluffy area, which extended in all directions in concentric circles. On microscopic examination, the mycelium was not coenocytic, the hyphae branched profusely and were septate particularly at their base. The cytoplasm was granular and vacuolate, particularly at the fast growing bulbous apex (Fig. 1).

Development of a black carbonaceous stroma was seen in the cultures in 8 to 22 days. The clusters of pycnidia in the stroma attained full development in 30 to 90 days. Pycnidia are usually immersed in the black stroma and are generally single chambered, although occasionally they have two or more chambers. Pycnidia are ostiolate, beaked or non-beaked, of a nearly globose shape (Fig. 2).

The conidiophores (Fig. 3) are simple and filiform with one celled spores which are of two types. The alpha spores are 3.8–8 μ long and 8.8–3.8 μ thick,

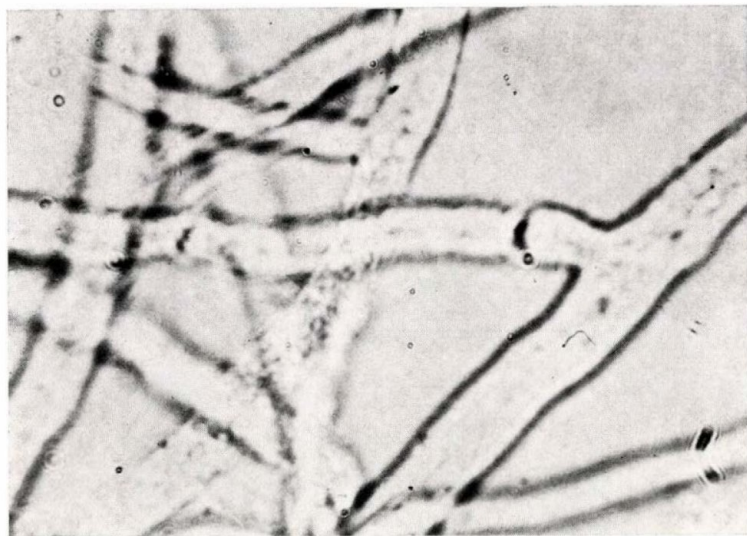


Fig. 1. *P. paspali*; septate mycelium



Fig. 2. *P. paspali*; pycnidium

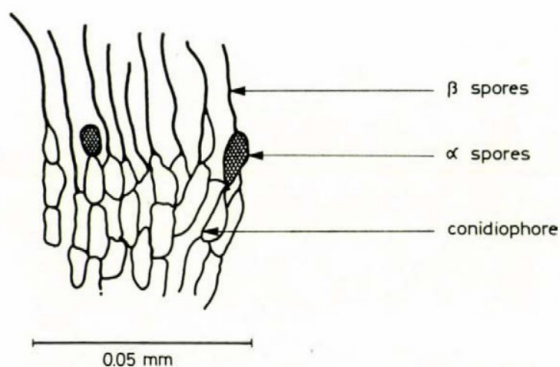


Fig. 3. *P. paspali*; conidiophores with α and β spores

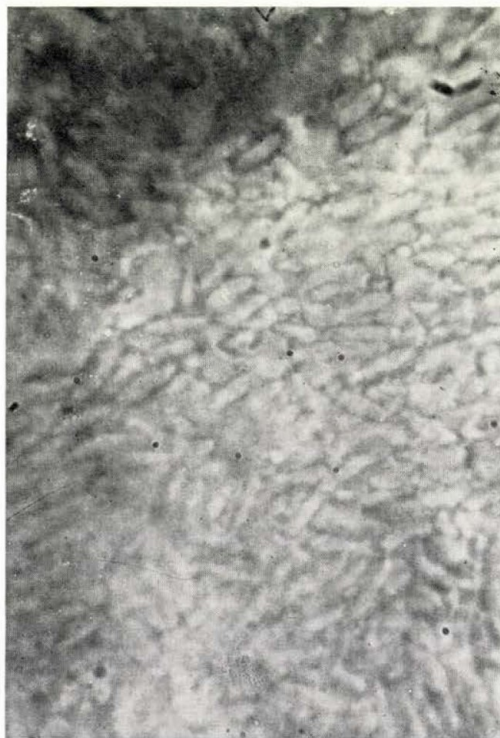


Fig. 4. *P. paspali*; α spores

ellipsoid, fusoid or oblong in shape, containing two small globules of oil in each spore (Fig. 4); this occasionally lends them a two celled appearance. The beta spores are filiform curved or bent (Fig. 5), 22.8–38 μ long and 1.0–3.5 μ thick (Fig. 3), mostly 22.6–24.4 μ long and 1.9–2.8 μ thick.

These data show that the fungal isolate belongs to class *Fungi imperfecti*, order *Spheropsidales*, family *Spheropsidaceae* and genus *Phomopsis* with



Fig. 5. *P. paspali*; β spores

the typical characteristics of non-coenocytic mycelium, conidiophores and conidia arising within pycnidia, hyaline conidia with fusoid and curved or bent shape, single celled, with simple conidiophores. It is the imperfect stage of *Diaporthe*.

SACCARDO [7] listed 84 species of *Phomopsis* all over the world, out of which VASUDEVAN [8] recorded 14 in India. A study of fungi prevalent in Maharashtra, which includes the region of the Western Ghats from where the present samples have been obtained, has been carried out by KAMAT *et al.* [9] in which three species of *Phomopsis* have been recorded. Table I summarizes the distinguishing characters of three species of *Phomopsis* commonly occurring in this part of the country, and their comparison with the present species.

It is seen from Table I that the present species is morphologically different from the others in the special characteristic of producing toxic metabolites, as well as in the host plant. This is the first time, that *Phomopsis* has been observed on *P. scrobiculatum* Linn.

Phomopsis paspali n. sp. Mycelium non-coenocyticum, hypha profuse divisum, septies radice rami, cytoplasmum granulame vacuolatum, pycnidia

Table I
Comparison of characters of different

Species	Host	Pycnidia types and dimensions
<i>Phomopsis vexan</i> Harter.	Leaves, stems and fruits of egg plant causing stem blight, leaf spot, and fruit rot & also damping off seedlings	Loosely gregarious compact at first, buried later, erumpent 60–200 μ in diameter on stems, 120–350 μ on fruits
<i>Phomopsis artabotrydis</i> Syd.	Living leaves of <i>Artabotrydis odoratissimi</i> (<i>Anonaceae</i>) forming spots 2–4 cm diam. ochrid, black or purple margin	Epiphyllie, compact, concealed, elevated, perforating the epidermis; flat, 150–200 μ in diameter
<i>Phomopsis pandani</i> , Died.	Leaves of <i>Pandanus</i> species (<i>Pandaneaceae</i>) spots elongated up to 10 cm; pale amphy-genis surrounded by yellowish and red line or ridge	Loosely gregarious, sub-epidermal, perforating the epidermis dorsally
<i>Phomopsis paspali</i> Pendse, Kanitkar n. sp.	Grain of <i>Paspalum scrobiculatum</i> Linn.	Stromatic, superficial or erumpent, 260–560 μ in diameter; pycnidium occasionally chambered

mersa in nigra stroma, simplex, singularis vel multicubiculata, ostiolata, rostrata vel non-rostrata cum forma fere globosa. Pycnidia erupta vel externa, conidiophorus simplex, filiform, spores unicubiculatae α et β .

α Spores ellipsoid, fusa vel oblonga, cum duobus parvis globulis α spores 3.8–8 $\mu \times 8.8$ –38 μ ; β spores 22.8–38 $\mu \times 1.0$ –3.5 μ . Grandis proportio 22.6–24.4 $\mu \times 1.9$ –2.8 μ .

The growth characteristics of the species on different solid and liquid media are summarized in Table II.

Carbohydrate utilization of the species was as follows. Glucose, maltose, raffinose, dextrin, fructose, saccharose were utilized well. Starch, laevulose and lactose were utilized moderately while cellulose was not attacked.

The ether extract obtained from ethanolic extract of the fungus, was subjected to thin-layer chromatography, which revealed three fluorescent and two non-fluorescent spots (Fig. 6). The non-fluorescent spots, being the major compounds present in the extract, were isolated first.

The non-fluorescent compounds I and II were isolated by column chromatography as described earlier. Their physical properties are given in Table III.

The result of micro-analysis is presented in Table IV. From mass spectral analysis (high resolution molecular peaks, 493.2838 for I and 457.2699 for II) and micro-analysis, the molecular formula for the two compounds was established as $C_{30}H_{39}NO_5$ and $C_{28}H_{37}NO_4$, respectively. Studies of the structure of these compounds are in progress.

Phomopsis sp. occurring in Maharashtra

Stylospores	Spores	Conidiophores	Reported toxic metabolites
Filiform, curved, 13–28 μ	5.8 $\times$ 2.8 μ , hyaline	Short, simple hyaline	Nil
Filiform, 6.5–8 μ	Fusoid hyaline, mostly biguttulate, 2–5.3 μ	Simple, filiform hyaline	Nil
Filiform, 7–9 μ	Fusoid, continuous, hyaline, biguttulate, 2–5.3 μ	Filiform, disintegrating sooner than sporules	Nil
Filiform, I shaped or S shaped, 22.8–38 $\times$ 1.0–3.5 μ	Hyaline, with 2 oil globules in each spore, 3.8–8 $\times$ 0.8–3.8 μ	Filiform	Paspaline PI and paspaline PII

Table II

Growth characteristics of Phomopsis paspali n. sp.

Medium	Growth*	Margin
<i>Agar media</i>		
(a) Potato dextrose agar (2%)	Rapid, thick, fluffy, aerial with distinct zones	Entire
(b) Malt extract agar (4%)	Rapid, thick, moderately fluffy, aerial with distinct zones	Entire
(c) Cohn's medium	Moderate without zones	Curled
(d) Coon's medium	Moderate, moderately fluffy and aerial without distinct zones	Undulate
(e) Uric acid medium	Slow, less fluffy without zones	Lobate
(f) Richard's medium	Moderate, fluffy and without zones	Undulated
(g) Czapek agar	Slow aerial, not fluffy and without zones	Lobate
<i>Nutrient liquid media</i>		
(a) Czapek	Moderate and slow	...
(b) Malt extract	Thick and rapid	...
(c) Richards	Fair, slow	...

* "Rapid" and "slow" refer to the rate of growth; "thick" and "moderate" refer to the extent of growth

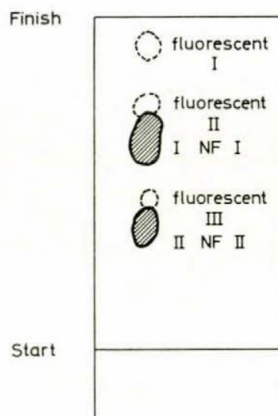


Fig. 6. Thin-layer chromatography of ether extract. Silica gel plates; solvent system, chloroform : methanol (95 : 5); spraying, 1% vanillin in 50% phosphoric acid

Table III

Physical properties of non-fluorescent compounds

Compound	Melting point	Solubility	Specific rotation	Molecular peaks at high resolution mass spectra
I	268 – 269	completely soluble in ether, alcohol, chloroform, methanol and Di-chloro-methane	$[\alpha]_{25} = +63^{\circ}$ D (c = 0.103, methanol)	496.2838
II	161 – 165° With heavy sintering at 147		$[\alpha]_{25} = +45^{\circ}$ D (c = 0.106, methanol)	451.2699

Table IV

Results of micro-analysis of compounds I and II

	I		II	
	Calculated	Found	Calculated	Found
C	72.99	72.64	74.47	74.48
H	7.96	7.87	8.26	8.21
N	2.84	2.77	3.10	3.04

Further collaborative work carried out and reported by PENDSE [10] revealed that the isolated compounds are new and closely related to cytochalasin D (zygosporeine A). They have been named paspalin-PI and paspalin-P. Compound I is now known to be the mono-acetate of compound II and

is hydrolyzed by K_2CO_3 in methanol/water at $25^\circ C$ in seven hours. This ensured an almost quantitative yield of the latter compound, thus showing a close similarity between the two compounds.

Preliminary studies of toxicity. Preliminary studies on the effect of the total ether extract in dogs and of the pure compounds in mice were carried out to observe the symptoms of poisoning and the lethal dose in mice.

Intraperitoneal injection of the ether extract in a dose of 6 mg/kg in a dog caused restlessness, nausea, vomiting and irritation followed by rigidity of hind limbs, swelling of the lower abdomen with dripping of saliva from the mouth. After two hours, the animal recovered.

The intraperitoneal injection of pure compounds I and II in mice was followed by marked effect on motor activity then a paralysis of the hind legs, depression of motor activity, convulsions and death. Table V summarizes the results and shows the lethal doses.

Table V
Lethal dose of compounds I and II in mice

Dose	No. of animals	Death	Remark
0.5 mg/kg	2	0/2	Motor activity depressed; recovery after 2 hr
2.0 mg/kg	2	2/2	Death within 45 min with convulsions
5.0 mg/kg	2	2/2	Death within 20 min with convulsions

Discussion

Paspalum scrobiculatum Linn. is one of the millets consumed in India. Many fungi have been reported on this host plant. However, the present isolate, characterized as a *Phomopsis* sp. has been designated *Phomopsis paspali* Pendse and Kanitkar n. sp. in view of the facts that it shows morphological differences from other species of *Phomopsis* reported in this region, and produces specific toxic metabolites not found in other species of *Phomopsis*. Moreover, this is the first time that *Phomopsis* sp. is reported on the host plant *Paspalum scrobiculatum* Linn. It cannot be said at this stage whether the toxicity of the plant is due to this or to other fungi reported earlier, or to something else.

The specific metabolites, suspected to be cytochalasins, make this species highly interesting and important, because these compounds are known for their important biological activities [11]. Further studies on the biological aspects of these compounds are in progress.

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EFFECT OF ARGININE DEFICIENCY ON THE REPRODUCTION OF HUMAN CYTOMEGALOVIRUS

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Summary. In arginine-deprived human embryonic fibroblasts the reproduction cycle of human cytomegalovirus (CMV) is incomplete. Infectious virus cannot be demonstrated in cell disintegrates, and from among the CMV antigens only the diffuse cytoplasmic antigen is detectable by immunofluorescence. The antigens localized in the cell membrane and those appearing during the complete cycle as large granules or inclusion-like bodies in the nucleus do not appear in the absence of arginine. The CMV genome persists in the arginine-deprived culture; after re-feeding with arginine-containing medium, maturation of virions soon ensues. Maturation could be prevented by inhibitors of protein synthesis, but not by DNA inhibitors, added simultaneously with completion of the medium.

Numerous papers have dealt with the effect of arginine deficiency on the reproduction of herpesviruses, *viz.*, herpes simplex virus (HSV) [1–10], Epstein-Barr virus (EBV) [11], Marek's disease virus [12, 13] and Aujeszky's disease virus [14], but its effect on the reproduction of cytomegalovirus (CMV) has only been mentioned in a short communication [15].

In the present work the effect of arginine deficiency on the reproduction cycle, including the appearance of virus-specific markers, of human CMV and the mechanism of the inhibition of virus replication was studied.

Materials and methods

Virus. The Rawles strain of human CMV; the strain has been characterized elsewhere [16].

Cell cultures. Primary and secondary human embryonic fibroblast cultures grown in Parker's 199 enriched with 10% calf serum (complete medium). In different experiments the serum and/or the arginine was omitted, or reduced in amount. To eliminate mycoplasma contamination, kanamycin (Resistomycin R Bayer, 1 g = 1.44 g kanamycin sulphate) was added to the medium [17].

Determination of virus yield. Human embryonic fibroblast monolayers were inoculated with 1 TCID₅₀/cell of virus. After an adsorption period of 1 hr the inoculum was removed; the monolayer was washed three times with PBS and then covered with a medium as indicated under Results. At the indicated intervals, the medium was poured off from three parallel cultures, the cells were thoroughly washed with PBS, trypsinized, resuspended, and made up to the original volume with complete medium. The suspension was homogenized by sonication (MSE ultrasonic apparatus, 1.5 A, 1 min) and centrifuged. The supernatant was titrated in tube cultures of human fibroblasts. The results were calculated according to the REED–MUENCH formula [18].

Indirect immunofluorescence (IF). To demonstrate intracellular and surface antigens, monolayers were fixed and stained as described elsewhere [19, 20]. Pooled serum from 10 CMV convalescents was used as antiserum. The anti-CMV titre of the pool was 1:256 as determined by the IF method.

Antiviral agents. Idouridine (IdU, 80 µg/ml); cytosine arabinoside (ara-C, 20 µg/ml); puromycin dihydrochloride (Sigma Chemical Co., St. Louis; 0.5 µg/ml); cycloheximide (Calbiochem, Los Angeles; 15 µg/ml).

Results

Arginine deprivation. (a) *The effect on virus synthesis of arginine-starvation of cell cultures before inoculation.* In preliminary experiments, virus synthesis was only slightly reduced in the absence of arginine from the maintenance medium of cultures grown in complete medium up to the time of inoculation. To reduce the arginine pool of the cells, the cultures to be infected were thoroughly washed with PBS and incubated in arginine-free medium for different periods of time before inoculation. A complete blockade of virus synthesis in the absence of arginine needed an arginine deprivation for 48 hr before inoculation. Thus, in the following experiments, all the cultures were covered with arginine-free medium for 48 hr prior to inoculation.

(b) *Effect on virus synthesis of serum in the arginine-free medium.* Varying amounts of serum were added to arginine-free Parker's 199 and the virus yield was measured on the 4th day post-inoculation. Table I shows that some virus reproduction ($10^{2.25}$) occurred even if only 1% serum was added to the arginine-free medium, but the yield was less than in complete medium, even in the presence of 10% serum. Since the effect of arginine deficiency was the most pronounced when the medium contained no serum, this was omitted from the maintenance medium in further experiments.

The special role of arginine in CMV replication. Human embryonic fibroblast cultures were deprived of arginine for 48 hr in Hanks' balanced solution (HBS), then inoculated with CMV and covered by a medium containing

Table I
Effect of different amounts of serum on cytomegalovirus synthesis

Serum concentration in medium, per cent	Virus yield by 4th day after inoculation (TCID ₅₀ /0.1 ml), in	
	Parker's 199	arginine-free Parker's 199
10	$10^{5.5}$	$10^{4.5}$
5	$10^{4.5}$	$10^{3.75}$
2.5	$10^{3.5}$	$10^{2.75}$
1.0	$10^{3.75}$	$10^{2.25}$
0	$10^{3.5}$	10^0

HBS + arginine or HBS + all essential amino acids except arginine. The virus yield was determined on the 4th day. Table II shows that a well-defined virus multiplication ensued in the absence of all amino acids except arginine whereas no, or hardly any, virus was recovered in the absence of arginine.

Effect on virus yield of the concentration of arginine. In these experiments serum- and arginine-free medium 199 was completed with different amounts of arginine and the virus yield was measured on the 4th day after infection. The half of the 70 mg/litre arginine present in medium 199 was sufficient,

Table II
Special role of arginine in cytomegalovirus replication

Medium	Virus yield by 4th day after inoculation (TCID ₅₀ /0.1 ml)
Hanks' solution + all essential amino acids except arginine	10 ⁰
Hanks' solution + arginine	10 ^{2.75}
Hanks' solution	<10 ⁰

but 17.5 mg/litre was insufficient for virus synthesis. Raising the arginine concentration to 140 mg/litre resulted in some rise in the yield, but a further excess in arginine did not change it.

Survival of the virus genome in arginine-deprived cells. Experiments were undertaken to show whether infectious virus could be recovered by completion of the medium with arginine from CMV-infected cultures having been incubated under arginine-free conditions. Arginine-free Parker's 199 was added to cultures immediately after infection and changed for Parker's 199 4, 6, 8 or 10 days later. Control cultures were incubated with Parker's 199 after infection and other cultures were incubated in the absence of arginine during the whole time of the experiment. Intracellular virus yield was determined in three parallel cultures on each day. For this purpose the cells were disintegrated. To count living cells cultures were stained with trypan blue.

Fig. 1 shows that virus could be recovered if the medium had been completed at any time during the first 10 days after inoculation. However, the virus titre was the lower the longer the starvation had lasted. The amount of recoverable virus was in direct relation to the number of living cells in the culture. Infectious virus was already demonstrable in the 24th hr, regardless of the duration of starvation.

Development of virus-specific antigens during arginine deprivation and after subsequent completion of the medium. Of the virus-specific markers, the

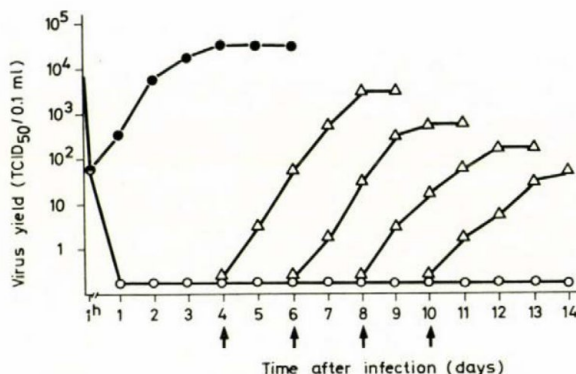


Fig. 1. Recovery of CMV from infected cultures whose medium was changed for Parker's 199 after an arginine deprivation of different duration. ●—● Parker's 199; ○—○ arginine-free Parker's 199; △—△ virus yield after re-feeding with Parker's 199; ↑ time of completion

intracellular antigens and those localized in the cell membrane were investigated.

In the absence of arginine, intracellular, mainly cytoplasmic antigens had developed. The nuclei appeared to be empty during the whole period of deprivation or there was fluorescence in about 40% of the nuclei; this localization is characteristic of early proteins [16]. In the nuclei, fluorescent granules or inclusion-like bodies were never seen. Virus-specific antigens were not detectable in the cytoplasmic membrane of living cells.

After re-feeding with Parker's 199, the membrane antigens appeared in the 12th hr, followed by intranuclear large granules and inclusion-like bodies which were detected in the 24th hr after completion, together with the infectious virus. Forty-eight, 72 and 96 hr after completion, membrane antigens were present in 85–95%, whereas nuclear antigens in 70–90%, of the cells. By the 96th hr the virus concentration had reached $10^{3.5}$ TCID₅₀/0.1 ml (Figs 2 and 3).

Mechanism of the inhibition of virus replication in arginine-deficient cultures. Human embryonic fibroblast cultures were inoculated with CMV and incubated with arginine-free Parker's 199 for 4 days. Then the medium was changed for Parker's 199 containing IdU or ara-C to inhibit DNA synthesis, or cycloheximide or puromycin to inhibit protein synthesis. Four-day incubation in the absence of arginine was chosen because by this time the cytoplasmic fluorescence had reached its maximum. The membrane antigens, the nuclear antigens appearing as large granules, and infectious virus were sought on the 4th day following completion, i.e., on the 8th day following inoculation (Table III).

The development of membrane antigens and of the nuclear antigens appearing as large granules was not influenced by the inhibitors of DNA

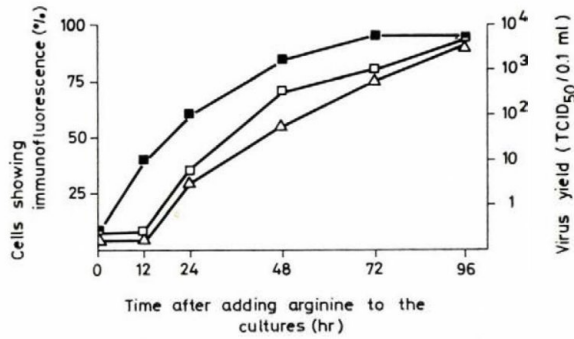


Fig. 2. Immunofluorescence and virus yield after completion of the medium with arginine. ■—■ Membrane antigens; □—□ inclusionlike antigens in the nuclei; △—△ infectious virus; 0 hr = time of completion

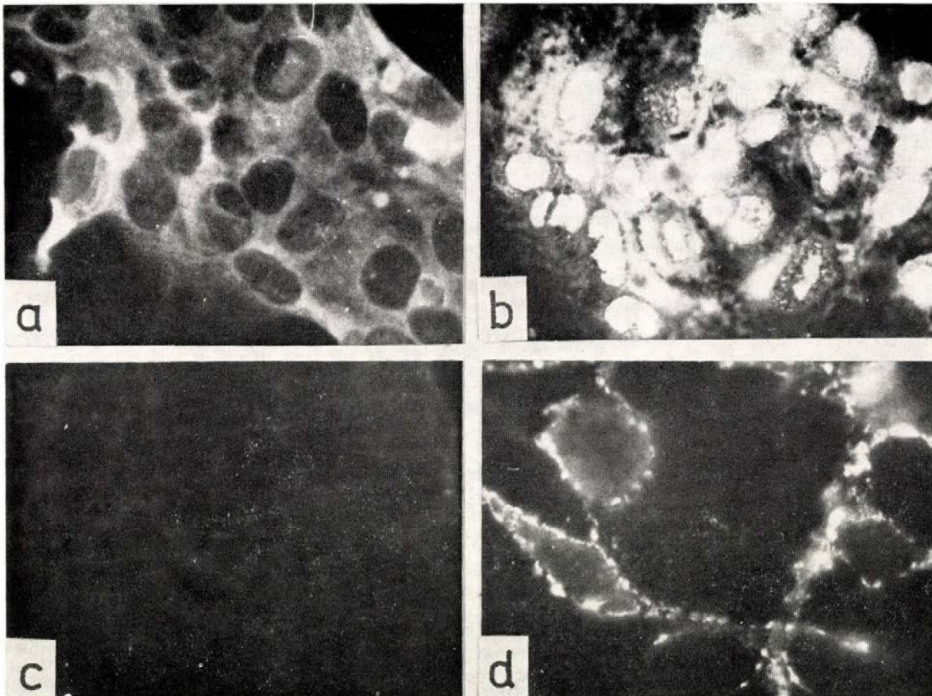


Fig. 3. (a) Cytoplasmic fluorescence of a culture incubated in arginine-free Parker's 199 for 4 days after inoculation with CMV. (b) Nuclear antigens on 2nd day after re-feeding with arginine-containing medium. (c) Membrane fluorescence of a culture incubated in arginine-free medium. (d) Membrane antigens on 2nd day after re-feeding with arginine-containing medium

Table III
Effect of arginine deficiency on viral DNA and protein synthesis

Culture No.	Medium		IF-positivity on 8th day after inoculation			Virus yield on 8th day after inoculation, log
	1-4 days	5-8 days	membrane IF	diffuse cyto- plasmic IF	fluorescent nuclear large granules and inclusion-like bodies	
	after inoculation					
1	—A*	—A	—	++++	—	—
2	+A**	+A	++++	++++	++++	10 ^{4.25}
3	—A	+A	++++	++++	++++	10 ^{3.5}
4	—A	+A +IdU	++++	++++	++++	10 ^{2.5}
5	—A	+A +ara-C	++++	++++	++++	10 ^{2.75}
6	+A +IdU	+A +IdU	++++	—	—	—
7	+A +ara-C	+A +ara-C	++++	—	—	—
8	—A	+A +cycloheximide	—	++++	—	—
9	—A	+A +puromycin	—	++++	—	—

* arginine-free Parker's 199

** Parker's 199

— no fluorescence

++++ approximately 90% of the cells showed fluorescence of the indicated type

synthesis given simultaneously with completion; even infectious virus was produced, though only about one-tenth of the yield of the cultures completed with arginine without inhibitor. On the other hand, in the presence of protein synthesis inhibitors, membrane antigens, nuclear antigens and infectious virus had failed to reach a detectable level.

In the cultures incubated with IdU- or ara-C-containing Parker's 199 from the time of inoculation, the development of membrane antigens was not inhibited but neither intranuclear large granules, not inclusion-like bodies and infectious virus were produced.

Discussion

The present results have proved the considerable dependence on arginine of human CMV.

The blocking effect of arginine deprivation was rapidly reversed by changing the medium for Parker's 199 at any time during the experimental

period. However, the amount of recoverable virus was the less the longer the cultures had been deprived of arginine (Fig. 1). The recoverable amount of virus was in direct relation to the number of living cells.

Some virus-specific proteins, first of all those diffusely present in the cytoplasm, appeared in the absence of arginine. These antigens, being inhibited by ara-C [16], may be considered structure proteins. The diffuse granules present in 30–40% of the nuclei in the infected cultures, on the other hand, are probably early proteins coded for by the DNA of the parent virus; their development is not prevented by ara-C [16].

Inhibitors of protein synthesis did, whereas inhibitors of DNA synthesis did not, prevent the synthesis of infectious virus in cultures re-fed with Parker's 199 after arginine starvation, suggesting that the proteins necessary for virus maturation are coded for by a DNA the synthesis of which does not require arginine.

MIKAMI *et al.* [12] claimed that arginine deprivation did not prevent the synthesis of viral DNA, but inhibited the development of the structural proteins of Marek's disease herpesvirus.

According to BECKER *et al.* [3], DNA synthesis of HSV proceeds normally in the absence of arginine. OLSHEVSKY and BECKER [6] reported that HSV capsid and envelope antigens are formed in the cytoplasm under arginine-free conditions and it is after re-feeding with Parker's 199 that they are transported into the nucleus to take part in the assembly of the nucleocapsid and in the envelopment of the virion. In their opinion, either the synthesis or the utilization of the viral core proteins is inhibited by arginine deficiency.

Other investigators, too, attribute a role to arginine in the migration from the cytoplasm to the nucleus of structure proteins. Selective inhibition of protein migration in the absence of arginine has been described for pseudorabies virus [14] and for HSV [5, 7–9].

The inhibition of the migration of structure proteins suggests that the synthesis of one of the proteins might intensely be inhibited in the absence of arginine. This protein might be identical with the virus core protein which, according to OLSHEVSKY and BECKER [6], could not be detected under arginine-deficient condition.

We have shown that, like other herpesviruses, the CMV is arginine-dependent. The inhibition of viral DNA synthesis cannot be responsible for the lack of virus synthesis, the latter being due to the inhibition of the synthesis of a protein (or proteins). The affected proteins must not represent the bulk of the structure proteins, for these are detectable under arginine-deficient conditions by their intensive fluorescence. The affected proteins may account for the migration of structure proteins from the cytoplasm into the nucleus.

The incubation of CMV-infected cells in arginine-free medium reminds of the latency following productive infection in multicellular organisms. The

latency, a phenomenon so characteristic of herpesviruses, may perhaps be related to the balance or imbalance of extra- and intracellular metabolites.

Acknowledgements. The authors are indebted to Mrs. J. HERPAY and Miss M. LOVÁSZ for excellent technical assistance.

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POST-THERAPEUTIC LEVELS OF ANTIMICROBIAL DRUGS IN FAECES

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Summary. After oral administration for 3 days, furazolidone disappeared from the faeces on the first, polymyxin B and ampicillin on the fourth, and trimethoprim + sulphamethoxazole and nalidixic acid on the fifth day.

In 1967 we reported on the persistence of microbiologically effective levels of antibiotics in faeces after therapeutic administration [1]. The present study gives an account of similar studies on other antibacterial agents.

From the intestine of adults, polymyxin B is absorbed in slight amounts or not at all [2]; about post-therapeutic faecal levels of this antibiotic there are only some non-quantitative data [3]. Ampicillin is absorbed slowly [4, 5]; for its faecal levels no data are available. Furazolidone absorption is practically nil and, due to inactivation in the intestine, only about 5% of the drug is excreted [6]. Trimethoprim + sulphamethoxazole (TMP + SMZ) is absorbed readily and thus the faecal level is low [7, 8]; the same is true for nalidixic acid [6, 9].

Materials and methods

The methods were the same as described in reference [1]. Modifications were as follows.

Test strains. For polymyxin B assay, *Bordetella bronchiseptica* ATCC 4617; for the assay of ampicillin, furazolidone and TMP + SMZ, *Staphylococcus aureus* ATCC 6538P; for nalidixic acid assay an *Alcaligenes faecalis* strain (sensitive to 0.5 µg/ml) isolated in our laboratory were used.

Media. Diagnostic Sensitivity Test Agar (CM 261, Oxoid) was used for all drugs except TMP + SMZ which was assayed on Welcotest Sensitivity Test Agar (Wellcome). For polymyxin B assays the medium was supplemented with 10 g polysorbate 80 per litre. The TMP + SMZ standard solution contained the two components at a ratio 1:5.

Administration of drugs. Groups, each containing 10 adults free from enteric symptoms, were treated orally for 3 days. The daily doses were: polymyxin B (Pfizer) 200 mg; ampicillin (Semicillin[®], Chinoïn, Budapest) 2, 3 or 4 g to 3 groups of 10 persons; furazolidone (Chinoïn) 800 mg; TMP + SMZ (Sumetrolim[®], EGYT, Budapest) 0.32 + 1.6 g; nalidixic acid (Nevigramon[®], Chinoïn) 4 g.

Results and discussion

Polymyxin B was present in the faeces at microbiologically effective concentrations on the first and second day after discontinuation of oral administration; by the fourth day it had disappeared from all persons examined (Table I).

Table I
Faecal level of polymyxin B after oral administration

Patient	Faecal concentration, $\mu\text{g/ml}$ on days after the end of treatment				
	1	2	3	4	5
1	100	50	0	0	0
2	20	0	0	0	0
3	100	50	20	0	0
4	20	0	0	0	0
5	200	20	0	0	0
6	200	20	0	0	0
7	150	100	0	0	0
8	150	50	0	0	0
9	200	50	0	0	0
10	50	0	0	0	0
Average	119	34	2	0	0

Results with ampicillin (Table II) allowed to conclude that an occasional excretion of the drug at effective concentrations was independent of the amount ingested.

As shown in Table III, TMP + SMZ disappeared from the faeces by the fifth day; on the fourth day 50%, on the third day the majority, of the patients still excreted the drug.

Nalidixic acid was present in two patient's faeces for four days (Table IV). Thus the drug is usually present at effective concentrations for 3 days after discontinuing therapy.

As expected from literary data [6], furazolidone disappeared rapidly from the faeces; even on the first day only 1 out of the 10 patients excreted it.

Thus, after discontinuing therapy, polymyxin B, nalidixic acid and TMP + SMZ persist in the faeces for a few days in antibacterial concentrations. Administration of these drugs may, accordingly, influence the result of bacteriological examination. As the persistence of faecal drug levels varies with the substance used, the interval between the end of therapy and sampling

Table II
Faecal level of ampicillin after oral administration

Patient	Daily dose, g	Faecal concentration, $\mu\text{g/ml}$ on days after the end of treatment				
		1	2	3	4	5
1—8	2	0	0	0	0	0
9		100	10	10	0	0
10		20	traces	0	0	0
1—8	3	0	0	0	0	0
9		50	0	0	0	0
10		140	110	0	0	0
1—7	4	0	0	0	0	0
8		30	0	0	0	0
9		30	35	traces	0	0
10		50	40	20	0	0

Table III
Faecal level of TMP + SMZ after oral administration

Patient	Faecal concentration, $\mu\text{g/ml}$ on days after the end of treatment				
	1	2	3	4	5
1	50	50	5	2	0
2	50	2	2	0	0
3	50	50	5	1	0
4	50	50	5	1	0
5	50	50	traces	0	0
6	50	25	5	traces	0
7	40	10	1	0	0
8	40	40	10	4	0
9	40	10	2	traces	0
10	35	10	5	1	0
Average	46	30	4	1	0

should be considered individually for each drug. In our opinion, for bacteriological control examinations in infectious diseases, the first faecal specimen should not be taken earlier than one day after the excretion of effective antibacterial drug concentrations has ceased. The proper interval between the end of

Table IV
Faecal level of nalidixic acid after oral administration

Patient	Faecal concentration, $\mu\text{g/ml}$ on days after the end of treatment				
	1	2	3	4	5
1	100	0	0	0	0
2	50	0	0	0	0
3	300	200	20	0	0
4	500	400	100	20	0
5	150	150	150	20	0
6	300	250	250	0	0
7	250	250	0	0	0
8	250	300	200	0	0
9	200	100	50	0	0
10	200	50	0	0	0
Average	230	170	67	4	0

therapy and sampling is estimated at 5 days for polymyxin B and ampicillin, and 6 days for nalidixic acid and TMP + SMZ. After furazolidone therapy, bacteriological specimens may be taken as soon as on the next day.

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VIRUS-SPECIFIC CHANGES IN MOUSE CELLS INOCULATED WITH A STRAIN OF HUMAN CYTOMEGALOVIRUS

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Summary. Cytopathic changes and virus-specific antigens developed in, then disappeared from, mouse fibroblasts infected by a strain of human cytomegalovirus (CMV), but their disappearance was delayed in cells treated with idoxuridine prior to infection. The replication of vesicular stomatitis virus and herpes simplex virus was restricted in human CMV-infected mouse cells as long as human CMV-specific antigens were present. Virus-specific antigens could be induced by treatment with idoxuridine or arginine deficiency in mouse cells which had previously turned "negative".

Human cytomegalovirus (CMV) has been known to be host species specific. Exceptions have been demonstrated in a few instances [1–3]. Studies on virus-specific changes in non-human cells by strains of human CMV have become interesting since ALBRECHT and RAPP [4] transformed hamster embryonic fibroblasts by human CMV and these cells induced tumours in hamsters.

Materials and methods

Virus. Human CMV strain Rawles was received from the virus laboratory of St. George's Hospital, London, in 1964. Since then the strain has been maintained in human fibroblast cell cultures in this laboratory. Its titre fluctuated between $10^{5.5}$ and $10^{6.5}$ TCID₅₀/0.1 ml. Herpes simplex virus (HSV), strain "HIL" and vesicular stomatitis virus (VSV) strain "Indiana".

Cell cultures. Secondary mouse and human embryonic fibroblast cultures. As complete medium, Parker's 199 enriched with 10% bovine serum was used.

Treatment with idoxuridine (IdU) prior to infection. Confluent monolayers were exposed to IdU (80 µg/ml) in culture medium during 3 days. Then the IdU was removed, the monolayers were washed three times in PBS and inoculated with 1 TCID₅₀ of virus/cell. After 1 hr adsorption, the inoculum was removed, the cultures were washed three times with PBS and complete medium was added to the cultures. Control cultures were not treated with IdU prior to infection.

Treatment with IdU at later stages of infection. Monolayers that had lost the virus-specific antigens were exposed to IdU (80 µg/ml) for 3 days. Then the IdU was removed, the cultures were washed three times in PBS and fresh medium was added for an additional 3 days. The reappearance of human CMV specific antigens was examined.

Arginine-deprivation. The medium was removed from monolayers of mouse cells that had lost the virus-specific antigens and then the cultures were maintained for 3 days in a medium consisting of arginine-free Parker's 199 + 1% bovine serum. Complete medium was then added to the monolayers for an additional 3 days. The reappearance of human CMV antigens was examined.

Determination of human CMV-specific antigens. Both intracellular and surface antigens were tested. For demonstration of intracellular antigens, cells growing on coverslips were fixed and stained, as described by GÉDER *et al.* [5]. Surface fluorescence was tested on washed unfixed coverslip preparations as described earlier [6]. The serum pool examined was obtained from 10 CMV-infected patients. The pool had an anti-CMV titre of 1:256 as measured by immunofluorescence.

Determination of infectious virus. Replicate culture monolayers were washed three times with PBS. The cells were removed from the glass with trypsin, then counted, homogenized by ultrasonic vibration (MSE ultrasonic apparatus; 1.5 A, 1 min) in culture medium, and centrifuged. The supernatant was titrated on human embryonic fibroblast cells seeded in Petri dishes. After 1 hr adsorption, the infected human fibroblast cultures were overlaid with culture medium containing 0.3% agar. On the 10th day, the cultures were fixed with formalin, then, after removing the agar overlay, stained with 0.1% crystal violet and the plaques were counted. For determination of infectious virus in intact cells, a suspension of cells was used as inoculum. The number of suspended cells in 1 ml is given under "Results".

Susceptibility of the cultures to superinfection with VSV and HSV. On the 2nd or 14th days after infection with human CMV, cultures were inoculated with 0.1 TCID₅₀ of VSV or HSV/cell. The infectious VSV yield was determined in mouse, and the infectious HSV yield, in human, fibroblasts. The titres were calculated according to REED and MUENCH [7].

Reagents. IdU and ara-C (Sigma Chemical Company, Missouri, U.S.A.) were dissolved in PBS.

Results

Adsorption of human CMV to and its penetration into, IdU-treated and untreated mouse cells, cytopathic (CP) changes, virus-specific antigens, infectivity of these cells as well as their susceptibility to HSV and VSV were studied. The inducibility of virus-specific antigens in infected cells which had already been seemingly negative were also examined.

Adsorption. Adsorption of human CMV to pretreated and untreated mouse cells was compared with the adsorption to human cells. Mouse and human cells were infected with a multiplicity of 1–2 TCID₅₀ virus/cell and the unadsorbed virus was titrated immediately and 1 and 2 hr after infection. The difference between the amount of unadsorbed virus in the supernatant measured immediately and 1 and 2 hr after infection represented the amount of adsorbed virus. There was no significant difference in the adsorption rates of human CMV to treated or untreated mouse and human cells (Table I). All of the treated and untreated mouse cells and human cells showed membrane fluorescence after 2 hr of adsorption (Fig. 1/a).

Penetration. Penetration of human CMV into pretreated and untreated mouse cells was then compared with its penetration into human cells. After 2 hr of adsorption the infected cells were washed three times with PBS and then treated with anti-CMV rabbit serum for 1 hr. Anti-CMV rabbit serum neutralized 100% of the inoculum in a separate experiment. Control cultures were treated with preimmune rabbit serum. After 1 hr, cells were again thoroughly washed with PBS, trypsinized, resuspended in culture medium of identical volume as the original and the virus in the supernatant of the disrupted cells was titrated. Treatment with antiserum reduced the amount of virus by 50% in every culture in comparison with the amount of virus

Table I

Adsorption of human CMV to treated and untreated mouse cells immediately after infection

Time of adsorption, hr	Percentage of virus* adsorbed to		
	untreated mouse cells	IdU-treated mouse cells	human cells
0	0	0	0
1	58	60	62
2	65	65	68

* The unadsorbed virus in the culture medium was titrated at intervals. The quantity of virus determined at 1 and 2 hr after inoculation was subtracted from the 0-hr value and expressed as its percentage

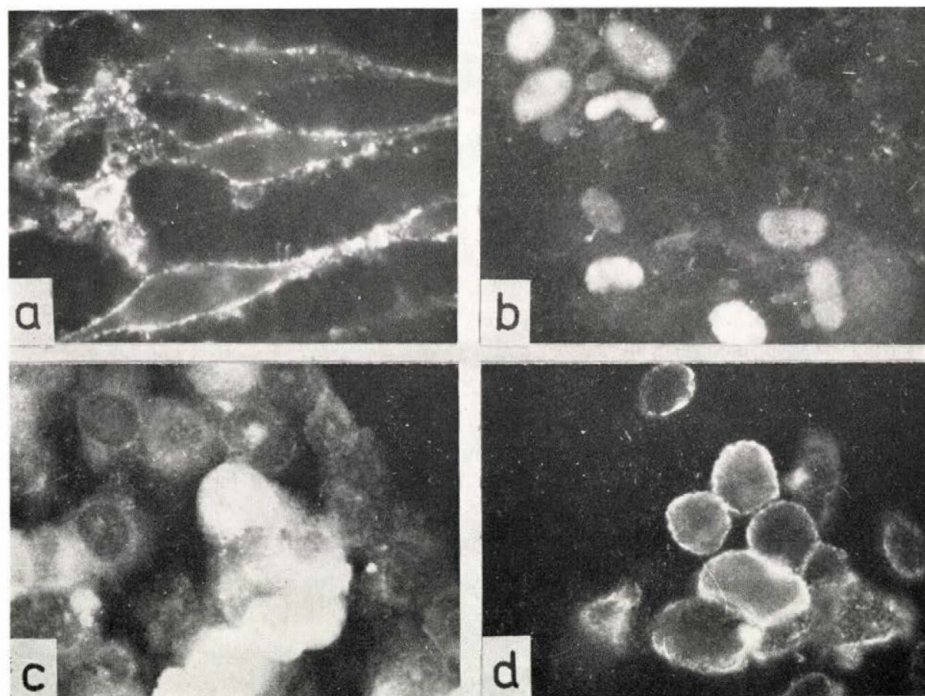


Fig. 1. Virus-specific immunofluorescence in mouse cells inoculated with human CMV; (a) membrane fluorescence 2 hr infection; (b) antigens diffusely distributed in the nucleus of pretreated cells 12 hr after infection; (c) antigens diffusely distributed in the nucleus and cytoplasm of pretreated cells 24 hr after infection; (d) surface antigens of pretreated cells 24 hr after infection

recovered from cultures treated with preimmune serum. These data indicate that during the 3 hr of incubation, 50% of the virus had penetrated into the treated and untreated mouse as well as human cells.

Cytopathic changes. Table II shows that untreated cells exhibited slight CP changes from the 1st to the 3rd day and these disappeared by the 5th day after infection. In pretreated cells the CP effect, characterized by the

Table II
Cytopathic effect of human CMV on treated and untreated mouse cells*

Time after inoculation, days	Mouse cells	
	untreated	IdU treated
1	55	60
2	35	65
3	20	80
4	10	80
5	2-4	50
6	0	35
7	0	10
8	0	0

* Expressed in the percentage of rounded cells

presence of rounded cells, was more intensive and disappeared only by the 8th day. Fig. 2 (a, b, c, d) show untreated mouse cells at the time of, and 1 day after, inoculation and pretreated mouse cells 1 and 3 days after inoculation. Inclusionlike bodies in the nucleus or in the cytoplasm were absent.

Virus-specific antigens. The data in Table III show that pretreatment with IdU increased the development and delayed the decrease of immunofluorescent antigens. At 12 hr postinfection, antigens diffusely distributed in the nucleus could be detected in 80% of IdU-treated cells (Fig. 1/b) and in 20% of untreated cells. Twenty-four hr after inoculation, nuclear fluorescence was the same as at 12 hr but fluorescence in the cytoplasm appeared in 90% of pretreated (Fig. 1/c) and in 60% of untreated cells. Membrane fluorescence was demonstrated in every cell (Fig. 1/d). In untreated cultures, maximum fluorescence was seen on the 2nd and 3rd days; the number of cells containing virus-specific antigens began to decrease on the 4th day and disappeared by the 7th-8th days after infection. In pretreated cultures, fluorescence reached its maximum on the 2nd day and began to decrease on the 8th day to disappear by the 14th day. The inhibition of virus DNA synthesis by the presence of 20 µg/ml ara-C did not influence the appearance and disappearance of antigens in pretreated as well as in untreated cultures.

Infectivity. Intact cells and the supernatants of disintegrated cells were examined for infectious virus by plaque titration. It can be seen from Table IV that infectious virus was detected from intact and disrupted untreated cells on the 1st–7th and 1st–3rd days, respectively. Intact mouse cells treated first with IdU and then infected with human CMV, contained infectious virus on the 1st–9th days and disintegrates of these cells had infected human cells

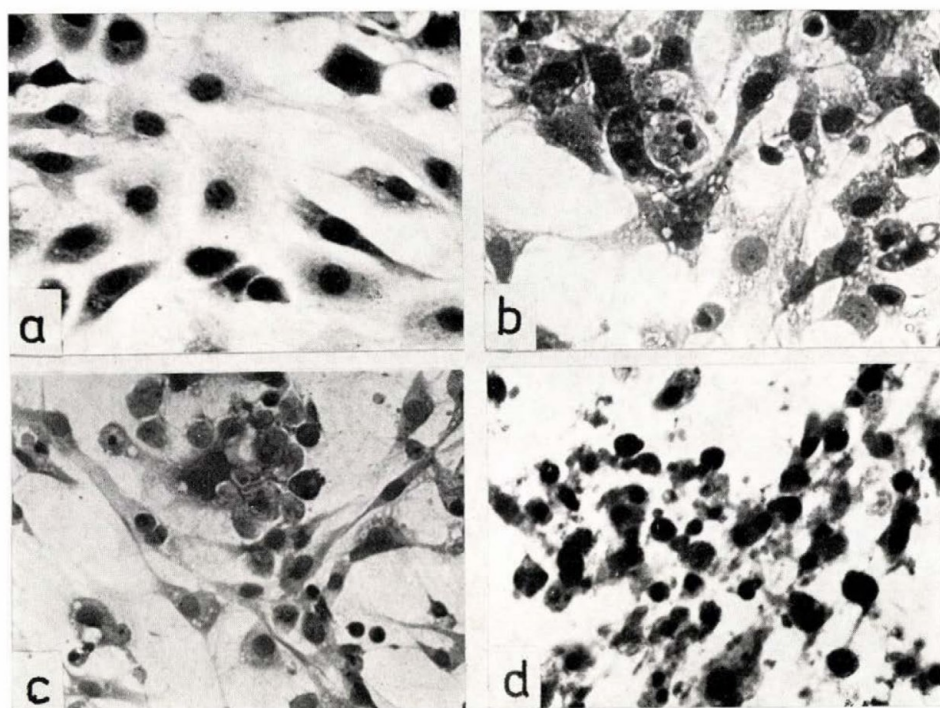


Fig. 2. Virus-specific CP changes in mouse cells inoculated with human CMV: (a) untreated mouse cells; (b) untreated mouse cells 1 day after infection; (c) pretreated mouse cells 1 day after infection; (d) pretreated mouse cells 3 days after infection

on the 1st–8th days after infection. The suspensions of cells pretreated with IdU contained fewer cells than those of the untreated cells.

Interference. As we found maximum fluorescence on the 2nd day and a complete disappearance of it on the 14th day after infection even in IdU treated cells, superinfection of cultures with HSV, known to replicate in the nucleus, and VSV, known to replicate in the cytoplasm, was performed at those points of time. Multiplicity was 0.1 TCID₅₀ virus/cell in both cases. The total yields of HSV and VSV were determined at the 24th and 48th hr after inoculation. Control cultures, treated or not treated with IdU, were not inoculated with human CMV. Fig. 3 shows that control cultures yielded infectious HSV and

Table III

Percentage of cells showing immunofluorescence in pretreated and untreated mouse cells, in the presence of, and without, ara-C

Time after inoculation, days	Immunofluorescent elements in											
	untreated cells						IdU-treated cells					
	without ara-C			with ara-C			without ara-C			with ara-C		
	Mem-brane	Nucleus	Cyto-plasm	Mem-brane	Nucleus	Cyto-plasm	Mem-brane	Nucleus	Cyto-plasm	Mem-brane	Nucleus	Cyto-plasm
0.5	100	20	0	100	20	0	100	80	0	100	80	0
1	100	20	60	100	20	60	100	80	90	100	80	90
2	100	50	100	100	50	100	100	100	100	100	100	100
3	100	50	100	100	50	100	100	100	100	100	100	100
4	100	10	70	100	10	70	100	100	100	100	100	100
5	45	5	40	45	5	40	100	100	100	100	100	100
6	10	1-2	10	10	1-2	10	100	100	100	100	100	100
7	1-2	0	0	1-2	0	0	100	100	100	100	100	100
8	0	0	0	0	0	0	100	80	90	100	80	90
9	0	0	0	0	0	0	100	70	75	100	70	75
10	0	0	0	0	0	0	80	40	60	80	40	60
11	0	0	0	0	0	0	50	35	40	50	35	40
12	0	0	0	0	0	0	15	10	10	15	10	10
13	0	0	0	0	0	0	1-2	0	0	1-2	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0

Table IV

Infectivity of treated and untreated mouse cells at different intervals after inoculation

Time after inoculation, days	Untreated cells			IdU-treated cells		
	cell number $\times 10^2$ /ml	Virus yield (p.f.u./ml) of		cell number $\times 10^2$ /ml	Virus yield (p.f.u./ml) of	
		intact cells	disintegrates of cells		intact cells	disintegrates of cells
1	120	19	4	110	20	4
2	140	12	3	125	16	4
3	150	11	4	70	11	3
4	120	8	0	55	12	4
5	100	8	0	40	15	4
6	120	7	0	40	12	3
7	130	2	0	25	2	1
8	110	0	0	24	2	1
9	90	0	0	20	1	0
10	90	0	0	25	0	0

VSV while human CMV-infected mouse cells did not replicate them, when cultures were superinfected with HSV and VSV on the 2nd day after human CMV inoculation. The interference was of low degree when human CMV-infected mouse cells were infected with HSV or VSV on the 14th day. Interferon could not be detected in the supernatant of human CMV infected mouse cells either treated or not treated with IdU, at any times after infection.

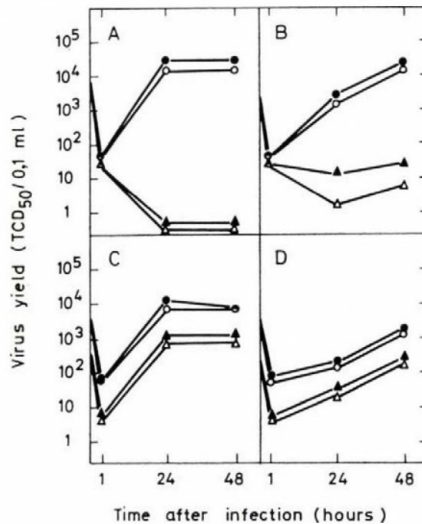


Fig. 3. Interfering effects of human CMV. A = infectious VSV and B = infectious HSV yields of mouse cells superinfected on the 2nd day of human CMV infection; C = infectious VSV and D = infectious HSV yields of mouse cells superinfected on the 14th day of human CMV infection; ▲—▲ pretreated and human CMV infected cultures; △—△ untreated and human CMV infected cultures; ●—● pretreated cultures; ○—○ untreated cultures

Inducibility of virus-specific antigens. The effect of IdU treatment and arginine withdrawal was then studied on the inducibility of virus-specific antigens in cells from which these antigens had disappeared. In these experiments, infected mouse cells not subjected to IdU pretreatment were used. As shown in Table V, treatment of infected mouse cells with IdU at times when the virus-specific antigens had already completely disappeared, resulted in the reappearance of these antigens. Treatment of mouse cells on the 10th, 20th or 30th day after infection gave rise to reactivation of the intracellular antigens in 50–70% and of membrane antigens in 10% of the cells. Imbalance of cell metabolism by withdrawal of arginine from the culture medium also gave rise to the reappearance of antigens. This phenomenon could be elicited during the whole period which the cells survived after infection, *i.e.* 30–40 days.

Table V

Percentage of cells showing immunofluorescence in mouse cells treated with IdU or by withdrawal of arginine at different intervals after inoculation

Time of IdU treatment or arginine withdrawal, days after infection	Immunofluorescent elements in								
	cells subjected to						untreated cells		
	IdU			arginine withdrawal					
	mem- brane	nucleus	cyto- plasm	mem- brane	nucleus	cyto- plasm	mem- brane	nucleus	cyto- plasm
10	10	50	70	10	50	70	0	0	0
20	10	50	70	10	50	70	0	0	0
30	10	50	70	10	50	70	0	0	0

Discussion

WANER *et al.* [2] published that infected Vero (heteroploid monkey kidney) cells and bovine embryonic skin-muscle cells infected by human CMV exhibited characteristic CP changes. FIORETTI *et al.* [3] noted CP changes and virus-specific antigens in guinea pig cells infected by human CMV; however a complete virus cycle failed to take place.

The effect of IdU treatment prior to infection on the replication of human CMV in epitheloid cells was examined by JEOR and RAPP [8]. Induction of virus-specific markers by IdU treatment or arginine deficiency in cell lines transformed by different viruses has also been studied [9–12]. The influence of IdU pretreatment on the replication of human CMV in cells derived from heterologous non-primate species and the induction of human CMV-specific antigens in heterologous cells which had lost their antigens, has not, however, been examined.

As there was no significant difference in the adsorption and penetration rates of human CMV into mouse and human cells and CP changes, virus specific antigens had developed, the CMV-restricted replication in mouse cells does not appear to be due to a failure of the virus to adsorb to and penetrate into mouse cells. The delayed disappearance of antigens and infectious virus from the cells pretreated with IdU might mean that a cellular product, which takes part in the disintegration of infecting virus, is depressed or coded for by a faulty mechanism in these cells. As viral infectivity was extremely low and decreased after infection, it is unlikely that new infectious virus was synthesized. This is in agreement with our observation concerning the antigens in the cells; the presence of ara-C did not prevent the development of these antigens, therefore they are regarded as early antigens coded for by parental viral DNA and not viral structure proteins.

The partial replication of human CMV in heterologous cells was proved by the inability of VSV to replicate in L cells infected by human CMV and by the inability of NDV to replicate in human CMV infected chicken cells [2]. In our system, beside the VSV, the interference was extended to HSV. Interference developed to such an extent on the 2nd day after CMV infection that pretreatment with IdU could not promote it. After the disappearance of human CMV antigens, the pretreated and untreated mouse cells regained the capacity to replicate VSV and HSV, thus regeneration of these cells was complete or near complete. Replication of VSV and HSV in cells pretreated with IdU but not infected with human CMV means that the metabolism of these cells is not disturbed inasmuch that replication of these viruses could be restricted.

Thus, the conclusion was that the replication of human CMV in mouse cells is prevented on the level of virus DNA synthesis.

The mechanism by which IdU and arginine deficiency activated the virus-specific antigens in cells that had turned "negative" is not known. Neither is it known whether the mechanism by which IdU pretreatment delayed the disappearance of antigens and by which IdU or arginine deprivation activated the antigens, have similar molecular steps.

We may, however, conclude that mouse cells infected by human CMV contained the virus genome, or some part of it, when virusspecific antigens and interference with VSV or HSV could not be demonstrated.

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ADENINE NUCLEOTIDE SYNTHESIS AND GROWTH RATE OF THE ADENYL SUCCINATE LYASE MUTANT OF *BACILLUS ANTHRACIS*

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Summary. After mutations affecting the *de novo* adenine nucleotide synthesis in *Bacillus anthracis*, the salvage pathway failed to ensure growth at the prototroph level. In sonicated suspensions of adenylyl succinate lyase deficient *B. anthracis* mutant, in the presence of exogenous 5-phosphoribosyl-1-pyrophosphate (PRPP), part of the labelled adenine was incorporated into adenine nucleotides. Addition of PRPP to cultures of adenylyl succinate lyase and adenylyl succinate synthetase mutants failed to promote growth. Both kinds of auxotroph showed *in vitro* a reduced growth rate as compared to the prototrophic strain.

There are several mutants derived from *Bacillus anthracis* non-capsulogenic strain Vollum [1]. Capsulogenic variants of adenine auxotroph mutants, although they produce toxin, are avirulent for the mouse [2, 3]. Part of the mutants are deficient in adenylyl succinate synthetase, part of them in adenylyl succinate lyase [2]. In both groups of adenine-requiring mutants the synthesis of alkaline phosphatase is partly derepressed [4]. The derepressed alkaline phosphatase synthesis could be repressed with adenine and with adenine nucleotides in the adenylyl succinate lyase mutant. Growth rate and ATP synthesis *in vitro* of this mutant were less than of the prototrophic strain [5].

Different catabolic enzymes such as adenosine deaminase and alkaline phosphatase seemed to decrease adenine nucleotide synthesis in the mutant [6]. In sonicated suspensions of the adenylyl succinate lyase deficient mutant, adenine nucleotide synthesis increased in the presence of exogenous ATP. Ribose-5-phosphate somewhat increased the stimulating effect of ATP [7]. Accordingly, in the mutant two pathways existed for adenine nucleotide synthesis from exogenous adenine. The ATP-requiring pathway involved nucleotide phosphorylase and adenosine kinase. In the other one, adenylic acid was formed in one step from adenine, using adenylyl phosphoribosyl transferase. The latter process required 5-phosphoribosyl-1-pyrophosphate (PRPP), while this had no direct effect on the reaction catalyzed by adenosine kinase. In view of these assumptions, it seemed desirable to examine the effect of exogenous PRPP on adenine nucleotide synthesis in, and growth *in vitro* of, the mutants.

Materials and methods

Substances. [^{14}C] adenine (Amersham), 500 $\mu\text{g}/27.9 \mu\text{Ci}$; PRPP (5-phosphoribosyl-1-pyrophosphate, Serva).

Bacterial strains. *B. anthracis* Vollum noncapsulogenic prototroph (VR) and its adenyl succinate lyase (23 *C⁻ ade⁻*) and adenyl succinate synthetase (344 *C⁻, ade⁻*) deficient mutants. The strains have been isolated and characterized by Ivánovics *et al.* [1, 2].

Medium. Lactalbumin hydrolysate, after removing phosphorus with barium hydroxide [8], was supplemented with different salts, amino acids, glucose, thiamine, adenine and K_2HPO_4 [4]; adenine concentration was 40 $\mu\text{g}/\text{ml}$, phosphate concentration 2.0 mM.

Cultivation. The complete medium was distributed in 10 ml aliquots into 100 ml Erlenmeyer flasks. Each flask was inoculated with 10^5 spores and aerated in water bath at 37°C. Optical density was checked by the Bausch and Lomb densitometer at 620 nm, once every hour.

Crude bacterial extracts (homogenates) were prepared by sonication as described previously [6] and their protein content was determined [9]. The extracts were used within 30 min after sonication.

Synthesis of [^{14}C]adenine nucleotides. The course of [^{14}C]adenine nucleotide synthesis in the sonicated suspension of the adenine auxotroph was examined under different conditions. To the crude bacterial extract, [^{14}C]adenine and PRPP were added. The experiments were carried out in 0.1 M cacodylate buffer (pH 7.4) containing 10^{-3} M MgSO_4 . The samples were incubated in 500 μl aliquots at 37°C. At intervals, 10 μl samples were removed and chromatographed on silica gel GF₂₅₄ thin-layer [6]. Activity of adenine nucleotides remaining at the start spot was determined in a Model 2001 Packard Tri-Carb Liquid scintillation spectrometer. The solvent was so chosen that the adenine nucleotide remained at the starting point while adenine, adenosine, hypoxanthine and inosine migrated at different R_F values [10].

Results

To examine the effect of PRPP on adenine nucleotide synthesis in the adenyl succinate lyase deficient mutant of *B. anthracis*, to the sonicated bacterial suspension (840 μg protein) 110 m μM [^{14}C]adenine and different amounts (0–400 m μM) of PRPP were added. The samples were incubated in water bath at 37°C for 20 min then 10 μl aliquots were chromatographed (Fig. 1).

These experiments clearly indicated the presence of adenyl phosphoribosyl transferase in the mutant. As the *de novo* biosynthetic pathway was damaged, the incorporation of labelled adenine into the adenine nucleotides depended on the amount of PRPP and of the enzyme.

The time course of adenine nucleotide synthesis was examined in sonicated suspensions in the presence of [^{14}C]adenine and PRPP. To the substrate solution containing 110 m μM [^{14}C]adenine and 110 m μM of PRPP, bacteria representing 820 μg protein were added. Samples were taken at 0, 20, 40 and 60 min and chromatographed for the activity of adenine nucleotides and of intermediary products of adenine metabolism. Fig. 2 shows that the amount of synthesized adenine nucleotides somewhat decreased while the inosine and hypoxanthine levels increased.

In subsequent experiments, the effect of exogenous PRPP on the growth of adenyl succinate lyase and adenyl succinate synthetase deficient mutants was examined. In these experiments it was considered that this compound

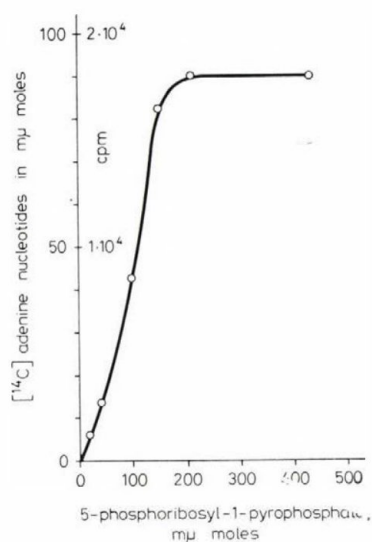


Fig. 1. Effect of PRPP on [^{14}C] adenine nucleotide synthesis in sonicated suspension of adenyly succinate lyase deficient mutant of *B. anthracis*; cpm values for 10 μl samples, m μM values for 500 μl samples

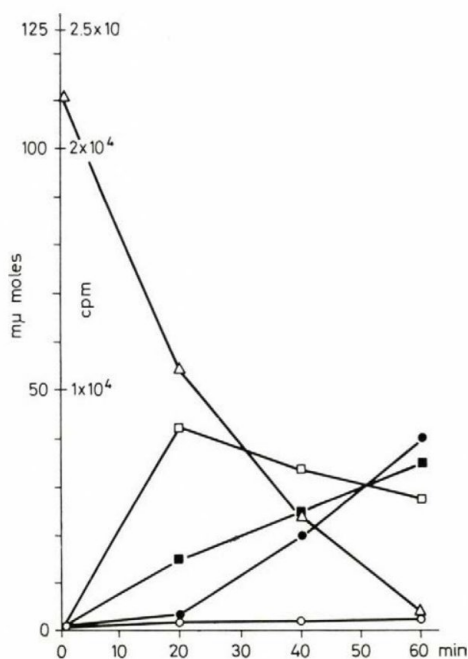


Fig. 2. Adenine nucleotide synthesis and metabolism in sonicated suspension of adenyly succinate lyase deficient mutant of *B. anthracis*; cpm values for 10 μl samples, m μM values for 500 μl samples; ●—● hypoxanthine, ○—○ adenosine, ■—■ inosine, □—□ adenine nucleotides, Δ—Δ adenine

might not be incorporated by the mutants. Since Gram-positive bacteria may take up large molecules such as haem [11], the effect of PRPP was examined. The lactalbumin medium contained 40 $\mu\text{g/ml}$ adenine, its phosphate concentration was adjusted to 2.0 mM. Under these conditions alkaline phosphatase synthesis in the mutants was repressed to a minimum, corresponding to that in the prototroph. The medium was inoculated with identical amounts of spores of the mutants and the prototroph and 2 mg PRPP were added to each of 10 ml aliquots of cultures in the early exponential phase (6 hr). During further incubation the optical density of the culture was determined at hourly intervals. It was shown that exogenous PRPP did not influence the growth of the mutants. The maximum optical densities were lower than the values for the prototrophic strain. The mutants failed to utilize exogenous PRPP *in vitro*, probably because the substance could not penetrate into the cells, or because it deteriorated rapidly. It would appear that both mutants exhibited a suboptimal growth.

Discussion

Mutation to adenylyl succinate lyase deficiency caused a loss of virulence in the capsulated variant of *B. anthracis*. The auxotrophic culture failed to multiply in the peritoneal cavity of mice even after the intraperitoneal injection of adenine. In smears prepared from the peritoneal exudate a gradual disintegration of the infecting cells was evident. A change in any of the structure genes involved in adenylic acid synthesis caused a loss of virulence by limiting the growth of the mutants. Guanine-requiring mutants were virulent after a supplement of exogenous guanine, although free guanine was not detectable in the peritoneal exudate [2].

Other experiments too indicated a disturbance in adenine uptake by adenylyl succinate lyase deficient mutants. Growth of the mutant *in vitro* stopped soon, while the medium still contained a considerable amount of adenine [7]. The adenylyl succinate lyase deficient mutant synthesized adenine nucleotides in the presence of exogenous PRPP, in other words, the organism produced adenylyl phosphoribosyl transferase. If adenine incorporation occurs in two steps (adenosine phosphorylase + adenosine kinase), PRPP exerts no influence on adenine nucleotide synthesis. The former of the two enzymes is a nucleotide phosphorylase; its activity is proven by the presence of hypoxanthine. The growth rate *in vitro* of the two adenine auxotrophs was lower than that of the prototroph. Growth of the auxotrophs was not promoted by PRPP, the cells were probably unable to take up exogenous PRPP.

Adenylic acid synthesis and growth of *B. anthracis* adenine auxotrophs depends on the amount of exogenous adenine and endogenous PRPP, if

adenyl phosphoribosyl transferase is present in sufficient amounts. *In vitro*, when adenine was added, the auxotrophs still exhibited a decreased growth; this may be attributed to a reduced growth ability of, or to a decreased level of PRPP or of the enzyme in, the mutants. No literary data are available for such a change in the enzyme level; it is, however, known that phosphoribosyl transferase activity is lower in the membrane of aged erythrocytes than of young ones [12]. The PRPP pool itself, as a precursor, may limit nucleotide synthesis from purine and pyrimidine bases [13]. Adenine auxotrophs require adenine for growth, but at the same time adenine inhibits to some degree the *de novo* synthesis of both purine nucleotides [14] and pyrimidine nucleotides [15]. It has been stated that the growth inhibitory effect of adenine can be eliminated with uridine [15] or with thiamine [16]. In the present experiments neither uridine nor thiamine given together with 50 μ g adenine improved the growth of adenine auxotrophs so as to reach the level of the prototrophic strain.

It may be assumed that the limited growth of *B. anthracis* adenine auxotrophs is due to a poor adenine nucleotide pool supplied by the salvage pathway.

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LIPID COMPOSITION OF THE MOUSE SPLEEN IN RAUSCHER LEUKAEMIA

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Summary. Lipid constituents were examined in the spleen of mice 10, 14 and 18 days after infection with Rauscher leukaemia virus. (i) The total lipid content, as related to the amount of protein, decreased with the development of the tumour. (ii) Spleen cells of control and infected mice synthesized the same lipids. (iii) In the infected spleen, free fatty acids increased by 80% and triglycerides decreased by 44% as compared to the control. The percentage distribution of phospholipid components showed a change in tumour cells: lecithin and phosphatidylethanolamine increased, lysolecithin and sphingomyelin decreased. (iv) After intraperitoneal injection of [^{32}P] orthophosphate, in infected mice the incorporation of ^{32}P into phosphatidylcholine and phosphatidylethanolamine increased, while ^{32}P incorporation into lysophosphatidylcholine + sphingomyelin and phosphatidylinositol + phosphatidylserine fractions decreased.

Membrane alterations are characteristic of cells transformed by viruses or chemical cancerogenic agents. According to the working hypothesis of HADJIOLOV [1], the pathologic features of the tumour cell are associated with an alteration of the membrane function. HOLLEY [2] attributed the development of tumours to a change in the cellular uptake mechanisms occurring as a result of disorders in the cell membrane, in consequence of which certain substances accumulate and induce rapid cell division.

Lipids are functional constituents of the membrane and, together with proteins, are responsible for its functional state. Lipids are needed for the normal function of certain membrane-bound enzymes, and for ensuring the normal permeability of the membrane.

Rauscher virus induces erythroleukaemia in Balb/c mice. The disease, in view of its short latency period, is an excellent model for studying the changes in the lipid metabolism of virus-transformed cells and the results obtained in this manner may contribute to a better understanding of metabolic changes in cells transformed by other RNA tumour viruses.

Materials and methods

Animals. Inbred Balb/c mice were infected with virus suspension prepared from the spleen of Balb/c mice suffering from Rauscher leukaemia. The spleens were homogenized in phosphate buffersaline to give a 20% suspension, then centrifuged at 4°C and 12 000 g. The mice were infected intraperitoneally with 0.2 ml supernatant.

The animals were given Mouse Feed (Institute for Breeding of Laboratory Animals, Gödöllő, Hungary) containing 92.3% dry material in the following distribution: raw protein, 22.2% (arginine, 0.95%; histidine, 0.6%; leucines, 3.6%; lysine, 1.2%; methionine, 0.5%; phenylalanine, 1.4%; threonine, 0.7%; tryptophan, 0.3%; valine, 1.6%); raw fat, 6.1%; raw fibres, 3.1%; N-free extract, 54.8%; ash, 6.1% (potassium, 0.7%; sodium, 0.2%; calcium, 0.9%; magnesium, 0.2%); vitamin A, 360 IU; vitamin E, 10.6 mg/100 g feed.

Lipids in the spleen were determined in normal and in leukaemic mice 10, 14 and 18 days after infection. The animals were exsanguinated, the spleen was removed and chopped with a dissecting needle in 0.83% ammonium chloride at 4°C. The cell suspension was pipetted off, left to stand at 4°C, decanted from the tissue debris, then centrifuged at 4°C and 200 g for 10 min. Washing with ammonium chloride was repeated twice in order to haemolyse the erythrocytes without damaging other cells [4].

The spleen of the normal mouse contains lymphoid (lymphoblast, lymphocyte) and lymphoreticular cells in 80–85%, erythropoietic and granulopoietic cells in 10–15% and some megakaryocytes. In leukaemic animals, 10–18 days after infection, the spleen suspension contained erythroblasts in 89–91% and proerythroblasts in 8–15%. Lymphocytes, lymphoid reticulum cells and polymorphonuclear leukocytes were present in less than 1%.

Lipid extraction. Cells washed in ammonium chloride were suspended in distilled water. The protein content was determined as described by LOWRY *et al.* [5], then the cells were extracted by PALMER's method [6]. The extracted lipids were washed as recommended by FOLCH *et al.* [7], then dried in inert atmosphere.

Raw lipids were separated into polar and neutral lipids by thin layer chromatography [8] and their ratio was determined gravimetrically from the isolated spots.

Phospholipides were separated by two-dimension thin layer chromatography on 500 μ Silicagel-G plates impregnated with 0.01 M sodium carbonate. Before use the plates were activated at 110°C for 30 min. The first dimension was run in chloroform: methanol: 25% ammonium hydroxide (65:25:4 v/v) until the solvent front had risen to 130 mm. Then the plates were dried with cool air for 15 min and exposed to concentrated hydrochloric acid vapour to remove excess ammonia. After a subsequent drying in cool air, the second dimension was developed with chloroform:methanol:acetone:95% acetic acid:water (72:15:30:15:7.5 v/v).

The dried plates were treated as described by VASKOVSKY and KOSTETSKY [9] and the components were identified by comparison to standards. Then the components were collected by scraping off the spots and the amount of phospholipides was determined by BARTLETT's method [10].

³²P uptake. [³²P]orthophosphate (OAB ³²P code P-RB-2 preparation) was injected intraperitoneally to mice at 2 μ Ci/g. Radioactivity of the phospholipids of spleen cells was determined 1, 2, 3 and 4 hr after injection. Raw phospholipids separated with two-dimensional chromatography were scraped off and measured together with the silicagel in toluene scintillation liquid in the Packard spectrometer. The method reflected 60–70% of the total activity.

Results

Examination of metabolic changes in tumour cells and evaluation of the results are made difficult by the fact that no adequate control cells exist. As an effect of Rauscher virus infection the cell constitution of the spleen exhibits a basic alteration. In our experiments, in addition to normal spleen cells, the liver was also used as a "control" of the phospholipid contents.

Data presented in Table I show no significant difference in the phospholipid content of normal spleen and liver. Accordingly, in subsequent examinations the neutral lipids and ³²P uptake were determined only in spleen cells. Fig. 1 presents a chromatogram of phospholipids extracted from normal cells.

As seen in Table I, the same phospholipids are present in Rauscher virus-infected and in normal cells. The quantitative differences are, in con-

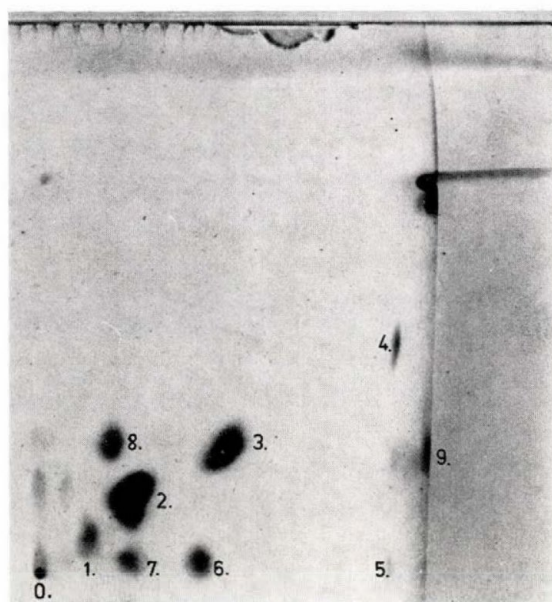


Fig. 1. Two-dimensional thin layer chromatogram of phospholipids extracted from the spleen of normal Balb/c mice. Silicagel G layer, 500 μ , impregnated with 0.01 M sodium carbonate. Dimension 1 (upwards): chloroform : methanol : 25% ammonium hydroxide (65 : 25 : 4); dimension 2 (from left to right): chloroform : methanol : acetone : acetic acid : water (72 : 15 : 30 : 15 : 7.5). Detection of spots: see reference [9]. Abbreviations: 0 = start, 1 = lysophosphatidyl-choline + sphingomyelin, 2 = phosphatidyl-choline, 3 = phosphatidyl-ethanolamine, 4 = diphosphatidyl-glycerol (cardiolipin), 5 = phosphatidic acid, 6 = phosphatidyl-serine, 7 = phosphatidylinositol, 8 = acylglycerophosphatidyl ethanolamine, 9 = ethanolamine plasmalogen

Table I

Percentage distribution of phospholipids isolated from the spleen of mice infected with Rauscher leukaemia virus

Phospholipids	Control		Spleen, days after infection		
	liver	spleen	10	14	18
Phosphatidylcholine	34.8	35.4	42.6	48.9	46.5
Phosphatidylethanolamine	13.8	13.9	19.4	16.8	19.8
Phosphatidylserine	7.19	8.9	6.4	5.5	5.7
Phosphatidylinositol	8.0	5.2	8.1	7.6	8.2
Diphosphatidylglycerol (cardiolipin)	2.4	1.0	1.6	2.3	1.9
Phosphatidic acid	2.1	0.4	0.1	1.2	1.2
Acylglycerophosphatidylethanolamine	9.0	12.0	11.8	7.5	7.4
Lysophosphatidylcholine + sphingomyelin	11.8	13.6	6.3	5.6	5.2
Other	11.0	9.3	3.4	5.3	3.7

Average of 3 parallel determinations

trast, evident: in infected cells the amount of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol was higher, while that of other phospholipids, especially of lysophosphatidyl-choline was lower as compared to the control.

The total amount of phospholipids and neutral lipids in infected spleen cells gradually decreased with the progression of the tumour; at the 18th day the total lipid, phospholipid and neutral lipid content amounted to about 50% of the control (Table II).

In addition to the decrease in the total amount of neutral lipids, the distribution of lipid groups was also different in leukaemic and normal cells: free fatty acids accumulated and triglycerides considerably decreased in the leukaemia cells. The ratio of cholesterol, cholesterol esters, mono- and diglycerides remained unchanged (Table III).

The accumulation of free fatty acids indicates a change in the metabolism of tumour cells.

Table II

Qualitative changes in lipid content of the spleen of mice infected with Rauscher leukaemia virus

Days after infection	Total lipid	Phospholipid	Neutral lipid
0	0.190	0.101	0.090
10	0.194	0.094	0.100
14	0.160	0.100	0.060
18	0.090	0.040	0.050

mg lipid/mg protein; average of 3 parallel determinations

Table III

Neutral lipid content in percentage of control in the spleen of mice infected with Rauscher leukaemia virus

Lipids	Days after infection	
	10	14
Triglycerides	78	56
Cholesterol esters	95	89
Cholesterols	117	108
Free fatty acids	189	171
Mono- and diglycerides	100	90

Average of 3 parallel determinations

Results of ^{32}P uptake experiments confirmed the data of quantitative analysis. Fig. 2 shows the percentage distribution of ^{32}P in the main phospholipid fractions of normal and infected spleen cells.

Seventy to 75% of the total activity was incorporated into phosphatidylcholine. In phosphatidylethanolamine 10–13% uptake was recorded. The activity in phosphatidylcholine reached the maximum in the first hour and showed no further change in the subsequent three hours. The percentage of ^{32}P incorporated into phosphatidyl-ethanolamine increased gradually during the four hour observation period. The ratio of activity measured in the phosphatidylinositol and phosphatidylserine fractions was the highest in the first hour, then decreased to 57% of the control.

^{32}P uptake by the lysophosphatidyl-choline and sphingomyelin fractions increased in the first three hours; in tumour cells only 1.25%, in normal cells 4%, of the total activity was demonstrated in these fractions.

Specific activities of different phospholipid fractions are presented in Fig. 3. In contrast to the percentage distribution of total activity, in specific activity there was a considerable difference between the phospholipids of normal and tumour cells. The difference was evident in the degree of activity as well as in the dynamics of uptake. In normal cells, maximum activity was

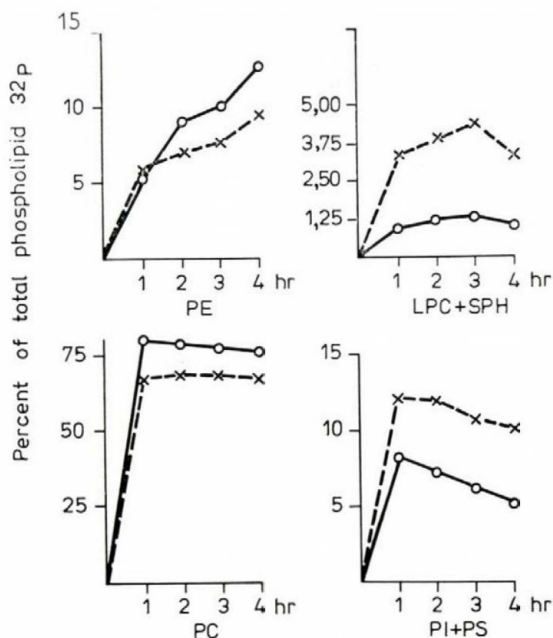


Fig. 2. Kinetics of ^{32}P incorporation into phospholipids of normal ($\times$ — $\times$) and transformed ($\circ$ — $\circ$) cells). Abbreviations: PE = phosphatidyl-ethanolamine, LPC + SPH lyso-phosphatidyl-choline + sphingomyelin, PC = phosphatidyl-choline, PI + PS = phosphatidylinositol + phosphatidyl-serine

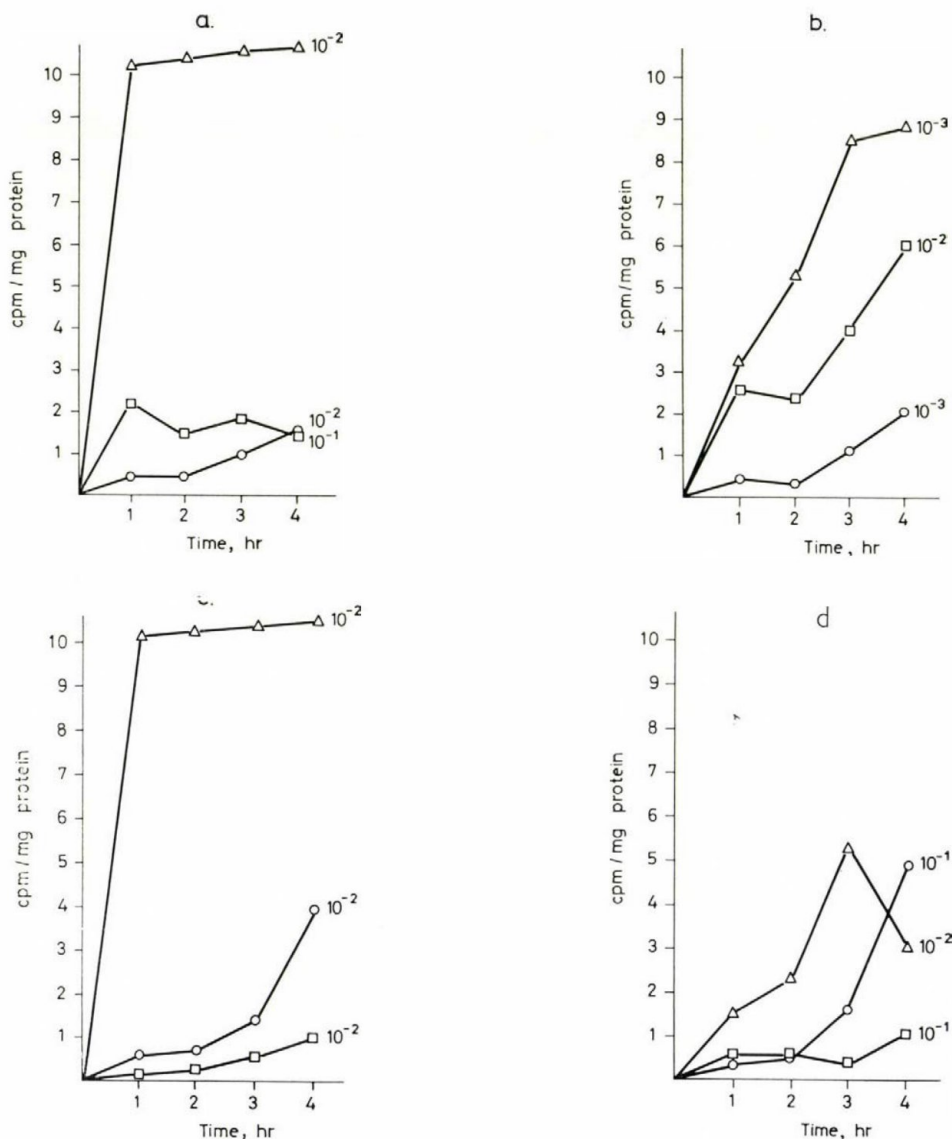


Fig. 3. Specific activity of phospholipid components; Δ — Δ control, $\circ$ — $\circ$ 10 days and $\square$ — $\square$ 18 days after infection; (a) phosphatidyl-ethanolamine, (b) phosphatidyl-choline, (c) phosphatidylinositol, (d) sphingomyelin

attained in the first hour in the phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine fractions. In tumour cells, ^{32}P incorporation increased gradually and reached no equilibrium during the 4 hour period. Tumour cells incorporated several times less ^{32}P than did normal cells. Maxi-

imum radioactivity in normal phosphatidylcholine and sphingomyelin fractions appeared in the third hour after injection of ^{32}P . Incorporation into the corresponding fractions isolated from tumour cells was considerably retarded in the first two hours, then increased, but by the fourth hour still no equilibrium had been reached. The activity of spleen specimens taken 18 days after infection was 100 times less than that of the control. The difference in ^{32}P uptake indicates a considerable reduction of the *de novo* synthesis of phospholipids in spleen cells of mice with Rauscher leukaemia.

Discussion

Spleen cells in normal and in Rauscher leukaemic mice were found to synthesize the same kinds of lipids. Quantitative difference in the components were, however, conspicuous: the total lipid content decreased with the progression of the tumour and within the total lipids the ratio of components was altered.

Our results confirm the data of DYATLOVITSKAYA *et al.* [11], who demonstrated that tumour cells and normal cells contained the same lipid components. In contrast, we could demonstrate a difference between tumour cells and normal cells in the distribution of the lipid components. According to PICIN and USONOV [12] the percentage ratio of lipid components was different in the normal lung tissue and in urethan-induced lung tumour of mice; ^{32}P uptake by tumour cell lipids was considerably less than in the control. Our data agree with those of ANGHILERI [13] who in different transplanted tumours found a low ^{32}P orthophosphate uptake into phospholipids, while ^{32}P phospholipids were incorporated in greater amounts and were stored for longer periods of time.

Literary data are contradictory as to the lipid content in definite groups of tumours. DYATLOVITSKAYA *et al.* [11] showed that transplantable tumours were of the same phospholipid composition as the rat liver. In the phospholipid content of mitochondria and microsomes there was, however, a qualitative difference: the selective phospholipid composition characteristic of normal cells was absent from tumour cells. FEO *et al.* [14] disagreed with the data of the above authors; in their opinion lipid metabolic changes in tumour cells were primarily characterized by an accumulation of cholesterol.

Our and ANGHILERI's data are in disagreement with those of MIRAS *et al.* [15], who claimed that in human chronic myeloid leukaemia cells, ^{14}C and ^{32}P uptake was increased both *in vivo* and *in vitro*.

The results of YAU and WEBERT [16] confirm that in tumour cells an altered lipid synthesis takes place. They found that in chick fibroblast transformed by Rous sarcoma virus the fatty acid content of phospholipids was significantly different from that in the controls. MITCHELL [17] showed that

the phospholipid content of the mitochondria of transplantable tumour cells was higher than that of mitochondria obtained from normal organs.

Recently, KOCH and DIRINGER [18] have shown a decreased phosphatidylinositol content of mouse fibroblasts transformed by SV₄₀ virus; they attributed this finding to a low enzyme activity in the cell membrane.

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STUDY OF *ESCHERICHIA COLI* O114 ASSOCIATED WITH A HOSPITAL OUTBREAK

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Summary. *Escherichia coli* O114 strains isolated from infants in the course of a hospital outbreak of enteritis were identical with type strain 26W in antigenic structure (O114:K90a, 90b), and in immunoelectrophoretic pattern (ORSKOV's group 1Bb) but differed from it in fermenting salicin late (18–22 days) and in phage pattern (group A₄); the strains produced colicin and were lysogenic. Pathogenicity of the organism was proved by IgM type antibodies in a patient's serum rising to a titre of 1:128 and by its enterotoxin-producing capacity shown by the rabbit ileal loop assay.

In May, 1973, an outbreak of enteritis was reported from the premature and infants' wards of the Country Hospital in Miskolc. In the course of the epidemic, 112 infants had enteric symptoms and 15 of them died. From the faecal specimens *Escherichia coli* O4 and O114 were isolated.

The role of *E. coli* O4 in enteric infections was described by NESTORESCU and POPOVICI [1], MALACHOWSKA [2], LINZENMEIER [3], BARON [4] and CZIRÓK *et al.* [5]. The pathogenicity of *E. coli* O114 was first mentioned by WRAMBY [6], who isolated this organism from enterotoxaemic calves. In 1952 BOKHARI and ORSKOV [7] included the organism in the Kauffmann–Knipschildt–Vahlne antigenic scheme under O114. The agent was isolated from human disease by ROGERS and CRACKNELL [8] and LINZENMEIER [3]. EWING *et al.* [9] regarded the members of this group as unfrequent pathogens and classified them into five serotypes. In Hungary, BARON [4] isolated *E. coli* O114 from the faeces of adults with gastroenteritis, and LAKATOS [10] gave an account of its incidence in the faeces of healthy and sick infants.

As *E. coli* O4 and O114 may both be responsible for enteritis, in the present study we have attempted to elucidate which of them had a role in the hospital outbreak in Miskolc.

Materials and methods

Faecal specimens were obtained from 15 infants with enteric symptoms and from 41 adults (nursing staff, mothers). A total of 56 specimens was examined. Colonies grown on eosin-methylene blue agar were transferred to agar slants, the growth was suspended in saline, heated at 100°C for 2 1/2 hr and tested by tube agglutination.

Serum production and serological examinations were performed as described by KAUFFMANN [11], EWING et al. [12] and EDWARDS and EWING [13]. Analysis of the antigenic structure of the isolates was carried out by the use of O and OK sera prepared from type strains 26W (O114:K90a,90b:H32) and U4/41 (O4:3L:H5) received from the State Serum Institute, Copenhagen, and from two of our isolates (M56897 and M56898).

Biochemical reactions were performed as described in reference [14].

Antibiotic sensitivity was examined with Resistest paper disks (Institute for Sero-bacteriological Production and Research Human, Budapest).

For *immunoelectrophoresis* the micromethod of SCHEIDEGGER [15] and a type 59951 OE 206 Labor MIM apparatus was used. Antigens were prepared as described by ØRSKOV et al. [16, 17], except that the bacteria were grown on meat infusion agar. The antigens were subjected to electrophoresis at a gradient of 6 V/cm with 0.02 M phosphate buffer pH 6.0 [18] on slides covered with 3.5 ml of 0.8% Oxoid No. 1 agar gel.

Antibody titres in 9 infant sera were determined by passive haemagglutination [19, 20].

Phage sensitivity, colicinogenic and lysogenic properties were examined as described previously [21].

Enterotoxin production was detected by the rabbit ileal loop assay [22-24].

Guinea pig eye reaction was tested as described by SERÉNY [25].

Results

Epidemiological and clinical observations. In the second half of May, 1973, a few cases of enteritis occurred in the newborn infants' wards. At the beginning of June, the number of infections increased to 2-3 cases daily and the same disease was reported from the premature babies unit. Mainly infants under four weeks of age were affected. After a 6-7 day incubation the disease began with 3-4 loose stools per day and subfebrility lasting for 3-4 days. In 80% of the cases a symptomless period of 4-5 days was followed by a relapse frequently characterized by toxic symptoms (anaemia, acidosis). For therapy and prevention first polymyxin-B then neomycin were administered. The patients received ample amounts of infusion [26].

Bacteriological cultures yielded *E. coli* O4 from 10 infants, *E. coli* O114 from 3 infants (two of these carried also *E. coli* O4). None of the examined adults (staff, mothers) showed the presence of these organisms.

Biochemical reactions. *E. coli* O114 isolates differed from the type strain in fermenting salicin only after 18-22 days.

Antigenic structures. Cross-absorption experiments showed that the isolates were identical with type strain 26W both in O antigen (O114) and in B-type K antigen (K90a, 90b).

Antibiotic sensitivity. The three O114 isolates were identical in antibiotic sensitivity pattern, whereas the O4 isolates exhibited different patterns.

Phage sensitivity. The *E. coli* O114 isolates belonged to the same phage type and phage group; the O4 strains showed different phage patterns.

Colicin production. The O114 isolates produced colicin acting on the same indicator strains. The *E. coli* O4 strains produced no detectable colicins.

Lysogenicity experiments. The *E. coli* O114 isolates were lysogenic and acted upon the same indicator strains. Of the 10 *E. coli* O4 strains, 9 were

Table I

*Phage sensitivity, colicin production and lysogenicity
of E. coli O4 and O114 strains*

Designation	Antigens	Phage pattern	Phage group	Colicin production (indicator strains)	Lyso- genicity	Source
W26	114: 90B: 32	—	nt*	Phi 17 K12	—	Type strain
M56897	114: 90B: .	12	A4	Phi K12	K12	Patient
M56898	114: 90B: .	12	A4	Phi K12	K12	Patient
M56899	114: 90B: .	12	A4	Phi K12	K12	Patient
U 4/41	4: 3L: 5	4	A1	—	—	Type strain
Bi 7457/41	4: 6L: 5	4, 17, 22	A1, B2, C1	17 K12	—	Type strain
Su 65/42	4: 12L: -	4, 20, 22	A1, B3, C1	—	—	Type strain
A 103	4: .: .	2, 3, 4, 12, 15, 22, PO	A1, A4, B1, C1, S	—	—	Type strain
M56890/1	4: .: .	4, (20)	A1, (B3)	—	—	Patient
M56891/1	4: .: .	—	nt	—	—	Patient
M56892/1	4: .: .	(4)	A1	—	—	Patient
M56893/1	4: .: .	4, (20)	A1, (B3)	—	—	Patient
M56895	4: .: .	—	nt	—	—	Patient
M56896/1	4: .: .	4, 20, (22)	A1, B3, (C1)	—	—	Patient
M56897/1	4: .: .	—	nt	—	—	Patient
M56898/1	4: .: .	—	nt	—	K12	Patient
M56900/1	4: .: .	—	nt	—	—	Patient
M56901/1	4: .: .	4	A1	—	—	Patient

* Non-typable

non-lysogenic. Phage sensitivity, colicinogenicity and lysogenicity of the isolates and of control type strains are shown in Table I.

Immunoelectrophoretic pattern of *E. coli* O114 isolates as compared to that of the type strain is presented in Fig. 1. The precipitation lines were identical with antigens heated at 60°C or at 100°C. As both kinds of extract showed arcs at the cathode side, the strains belonged to ØRSKOV's group 1Bb.

Pathogenicity tests. The isolates failed to cause keratitis or conjunctivitis in the guinea pig's eye. One *E. coli* O4 strain and one O114 strain were examined by the rabbit gut loop assay; the O114 strain gave a positive reaction.

Antibodies in patients. Nine infants were examined for antibody titres; one of them gave a positive faecal culture for *E. coli* O114, one harboured both O4 and O114, and one was negative for these organisms; from the re-

maining 6 infants no faeces were received for bacteriological examination. The haemagglutination titres ranged between 1:2 and 1:6 for *E. coli* O4, and between 1:2 and 1:20 for *E. coli* O114, with the exception of a 1-month-old baby, who showed at 1:128 titre for O114 and excreted the organism

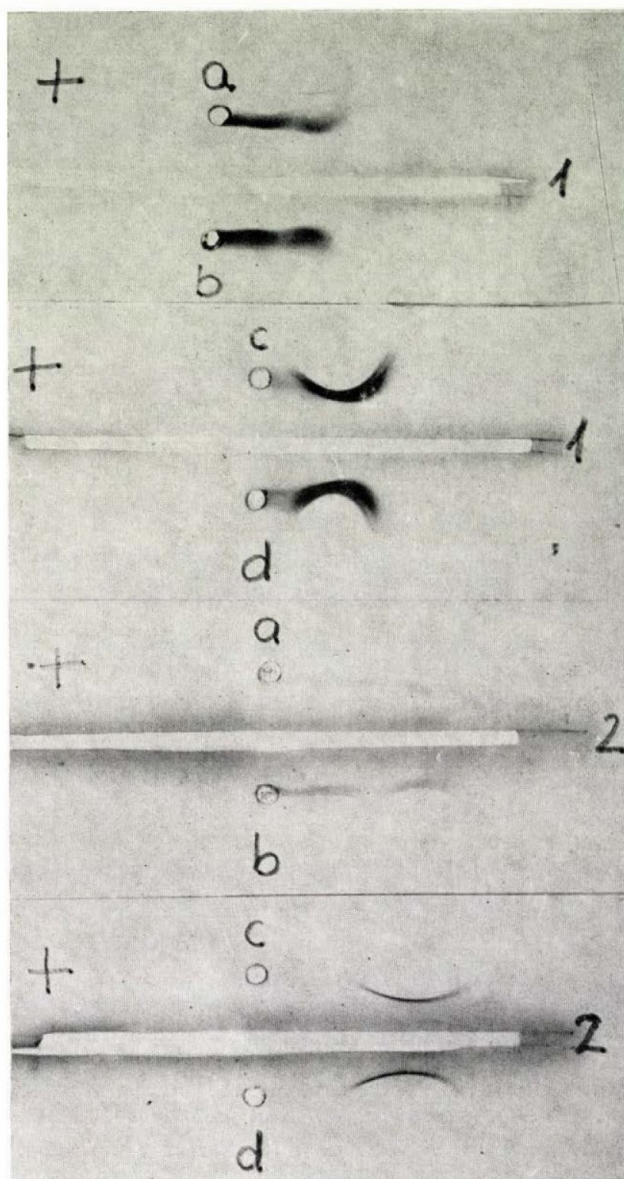


Fig. 1. Immunoelectrophoresis of *E. coli* O114 strains. Antigen M56899, 60°C, 20 min (a); antigen 26W, 60°C, 20 min (b); antigen M56899 100°C, 60 min (c); antigen 26W, 100°C, 60 min (d); OK serum M56899 (1); O serum M56899 (2)

in great numbers in the faeces. At a subsequent examination the titre decreased (Table II). After treatment with 0.02 M 2-mercaptoethanol the titre of the first serum sample decreased from 1:128 to 1:8.

Table II
Haemagglutinating antibodies to E. coli O4 and O114
in a 1-month-old infant

Time of sampling	Antibody titre	
	<i>E. coli</i> O4	<i>E. coli</i> O114
August 1, 1973	—	1:128
October 2, 1973	—	1:32
October 25, 1973	—	1:16

Discussion

The hospital outbreak described in this paper was somewhat similar in course to the 1951–52 Birmingham outbreak of *E. coli* O114 enteritis described by ROGERS and CRACKNELL [8]. Another severe epidemic with 30 deaths occurred in Manchester in 1969 [27], where the same serogroup of *E. coli* was isolated.

Bacteriological examination of our hospital outbreak yielded *E. coli* O4 and O114 strains. The responsibility of O114 for the epidemic was suggested by the finding that, unlike O4 strains, O114 isolates were uniform in antibiotic sensitivity, phage pattern and colicinogenicity, as well as by the fact that the latter produced enterotoxin while the former failed to do so. At least in one patient, against O114 there was a significant increase in antibody titre which decreased after convalescence. Mercaptoethanol sensitivity of the antibodies indicated IgM type factors produced actively in the new born infant.

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EFFECT OF MYCOPLASMA GALLINARUM ON THE REPLICATION IN VITRO OF GOOSE PARVOVIRUS STRAIN "B"

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Summary. Replication *in vitro* of the goose parvovirus strain "B" was inhibited by *Mycoplasma gallinarum* co-infection. This effect could be prevented by continuous supplementation of cell cultures with arginine. The infection of cell cultures with *Acholeplasma axanthum* did not influence virus replication.

In a previous paper we have described the isolation of *Mycoplasma gallinarum* from goose embryo fibroblast cell cultures prepared from seemingly healthy embryos [1]. The cytopathic effect (c.p.e.) caused by *M. gallinarum* in both goose and chicken embryo fibroblast cell cultures was characterized by severe cytoplasmic vacuolization and cytoplasmic inclusion bodies [2].

The arginine-metabolizing mycoplasma species are known to affect unfavourably the replication of some viruses *in vitro* [3]. Adenoviruses require for maturation arginine in the medium, therefore, in tissue cultures contaminated with arginine-metabolizing mycoplasmas, this step of virus replication will be suppressed [4, 5]. Type A influenza virus and vaccinia virus also require arginine for their replication [6, 7]. The synthesis of herpesviruses likewise seems to be dependent on the presence of arginine in the culture medium [8, 9]. The viral proteins of pseudorabies virus synthesized in the cytoplasm of the infected cells fail to migrate to the nucleus for assembling when the medium is lacking arginine [10]. In consequence, the synthesis of herpes simplex virus is suppressed if the tissue culture is contaminated with arginine-metabolizing mycoplasmas [11]. Recently, it has been reported that microplaque formation by varicella virus, a member of the herpesvirus group [12], was almost completely inhibited in tissue cultures infected with *Mycoplasma arginini* [13].

The present study had the aim of detecting whether the replication *in vitro* of goose parvovirus strain "B" [14] was influenced by arginine-metabolizing mycoplasmas.

Materials and methods

Tissue culture. Fourteen-day-old goose embryos were used for the preparation of goose embryo fibroblast cell cultures by methods described previously [15]. The cells were seeded into plastic Petri dishes (60 mm).

Virus strain. Goose parvovirus strain "B" [14] was employed.

Mycoplasma medium. The mycoplasma and acholeplasma strains investigated were cultivated in medium B [16] without thallium acetate and the horse serum was replaced by PPLO Serum Fraction (Difco).

Mycoplasma strains. *M. gallinarum* as arginine-metabolizing [17] and *Acholeplasma axanthum* as arginine non-metabolizing [18] strains were used.

Arginine. Arginine hydrochloride solution (24 mM) was prepared in double-distilled water and adjusted with NaOH to pH 7.2.

Assay. The cell suspension in plastic Petri dishes was infected with single or combined cultures of "B" virus strain (100 TCID₅₀), *M. gallinarum* (10%/0.1 ml c.f.u.) and *A. axanthum* (10%/0.1 ml c.f.u.) as shown in Table I. The proper cultures were supplemented daily with 0.2 ml per plate of 24 mM arginine hydrochloride solution. After 5 days incubation the cultures were frozen and thawed three times and centrifuged. The supernatants were mixed with chloroform in equal volume, kept at 37°C for two hr, centrifuged, and the supernatants were assayed for infectivity.

Results

As it is seen in Table I, infection of cell cultures with goose parvovirus strain "B" and *M. gallinarum* resulted in a suppression of replication. The effect could be prevented by adding arginine to the culture medium. Similarly, the c.p.e. characteristic of *M. gallinarum* failed to develop in cultures continuously supplemented with arginine.

Table I

*Effect of M. gallinarum on replication in vitro
of goose parvovirus strain "B"*

Cell cultures infected with	c.p.e.		Virus infectivity TCID ₅₀ /0.1 ml
	virus	mycoplasma	
"B" virus strain	+	·	10 ⁵
"B" virus strain + arginine	+	·	10 ⁵
"B" virus strain + <i>M. gallinarum</i>	NC	NC	10 ²
"B" virus strain + <i>M. gallinarum</i> + arginine	+	—	10 ⁵
"B" virus strain + <i>A. axanthum</i>	+	+	10 ⁵
"B" virus strain + <i>A. axanthum</i> + arginine	+	+	10 ⁵
<i>M. gallinarum</i>	·	+	·
<i>M. gallinarum</i> + arginine	·	—	·
<i>A. axanthum</i>	·	+	·
<i>A. axanthum</i> + arginine	·	+	·

NC = not characteristic

No inhibition of virus replication occurred in cultures simultaneously inoculated with *A. axanthum* and goose parvovirus strain "B". The development of c.p.e. characteristic of *A. axanthum* infection could not be prevented by supplementing the cell cultures with arginine.

Discussion

Replication *in vitro* of goose parvovirus strain "B" was suppressed by arginine-metabolizing mycoplasmas. This effect could be suspended by supplementing the cell cultures with arginine. The development of c.p.e. due to *M. gallinarum* infection could also be prevented by adding arginine to the culture medium. This finding is in good agreement with that described earlier in connection with murine lymphoma cells infected with mycoplasmas [19].

It was also observed that *A. axanthum* had no effect on the replication *in vitro* of goose parvovirus strain "B". Furthermore, the arginine-supplementation of cell cultures infected with *A. axanthum* did not influence the appearance of c.p.e. caused by this organism. It is, therefore, supposed that there is no competition for arginine as an essential metabolite between the goose parvovirus strain "B" and *A. axanthum*.

It is concluded that arginine must be essential for the replication of goose parvoviruses. Further studies are needed to clarify the nature of the phenomenon.

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THE MUTAGENIC EFFECT OF PESTICIDES ON *ESCHERICHIA COLI* WP2 *try*⁻

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Summary. Thirty pesticides commercially available in Hungary and three well-known chemical mutagens were applied to agar plate cultures of *hcr*⁺ and *hcr*⁻ derivatives of *Escherichia coli* WP2 *try*⁻ strain. After incubation, the plates were examined for a relative increase in reverse mutation number. N-Trichloro methylthio-1,2,3,6-tetrahydrophthalimide, dimethyl-2,2-dichlorovinyl-phosphate and N-trichloro methylthiophthalamide proved to have definite mutagenic activity.

The extensive use of an increasing number of chemicals in agriculture and the ensuing contamination of food by an increasing variety of pesticide residues raises several problems. In addition to the dangers of acute and chronic intoxication, the potential mutagenic effects of such contaminations could threaten the genetic health of coming generations and it is, therefore, of high importance that these effects be studied.

The present report describes the effects of a number of pesticides registered for use in Hungarian agriculture [1], and of well-known chemical mutagens, on reverse mutation in the *hcr*⁺ and *hcr*⁻ derivatives of *Escherichia coli* WP2 strain, using the spot test technique of IYER and SZYBALSKI [2].

Materials and methods

Strains. The bacterial strains used were the *hcr*⁺ and *hcr*⁻ (host cell reactivation deficient) derivatives of an auxotrophic mutant of *E. coli* WP2 *try*⁻. Strain WP2 *hcr*⁻, termed WP2 *uvrA* [3], differs from *hcr*⁺ in lacking one or more enzymes in its repair system which is active after UV-radiation [4]. The tryptophan requirement is due to an ochre nonsense mutation [5, 6] at a stage before anthranilic acid in the tryptophan synthesis pathway [7]. Mutations to non-requirement are due either to reverse mutations at the tryptophan locus or the induction of ochre suppressors [8]. No attempt was made to distinguish between these two types of back-mutation.

Media and growth. Cells were grown overnight at 37°C in mineral salts medium [9] supplemented with 50 µg ml⁻¹ of tryptophan and with 4 mg ml⁻¹ of glucose.

Spot tests were performed as follows. Overnight cultures were washed twice and resuspended in mineral salts medium to give a final concentration of approximately 10⁹ bacteria ml⁻¹, then 0.1 ml of this suspension was seeded on agar plates containing mineral salts, glucose, plus 1 µg ml⁻¹ of L-tryptophan. The agar surface was allowed to dry for 60 min, then sterile filter paper disks 12 mm in diameter were placed on each agar plate. The chemicals to be

tested were added to each disk either as a single 1–3 mg crystal or as a 20–25 μ l microdrop. Each compound was tested in triplicate and the plates were incubated at 37°C for 2 days.

During incubation the substance diffuses through the medium producing a concentration gradient, and revertant colonies induced by the chemical form a concentric circle around the disk, while the occasional spontaneous revertants are randomly distributed over the plate.

Two controls were run on each group, the standard control giving the number of spontaneous per 5×10^8 plated cells, in the case of *hcr*⁺: 30.5 ± 3.1 and *hcr*[−]: 32.2 ± 8.6 , the positive control with known mutagens demonstrating the mutagenic responsiveness of these particular strains.

Test compounds. The pesticides tested with *E. coli* WP2 (*hcr*⁺ and *hcr*[−]) are shown in Table I. Novenda, Anthio 25 EC, Aktikon, Dikonirt, Aresin (Nitrochemical Industries, Hungary); Tedion V-18 (Philips-Duphar, The Netherlands); Karathane, Dithane M-45 (Rhom and Haas, France); Orthocide 50 WP, Ortho-Phaltan (Chevron Chem. Co., England); Tenoran, Dimecron 50 (Ciba-Geigy AG, Switzerland); Sevin (Union Carbide, U.S.A.); Unifos (Universal Chemical Cooperative, Hungary); Lebaycid (Bayer, G.F.R.); Phosphothion (Dimitrov Chemical Industries, Czechoslovakia); Basudin, Buvinol, Hungaria, Ditrifon 50 WP (Chemical Industries of Budapest, Hungary); Sumithion 50 EC (Sumitomo, Japan); Vapam (Minoc, France); Zineb (Copci, France); Granosan (Federalchemexport, U.S.S.R.); Dymid 80 WP, Gardona 50 WP (Elanco Products Co., England); Bercema-ferbam 50 (VEB Chemia, G.D.R.); Cosan (Riedel de H  en, G.F.R.); Pol-Thiuram (Azot Chemical Industries, Poland); Gomex (Alkaloida Chemical Industries, Hungary) were supplied by the University of Horticulture, Budapest, Hungary. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was obtained from Fluka AG, Switzerland; N-nitroso-N-methylurethane (NMUr) from Pflatz and Bauer Inc., U.S.A.; Acridiniumchlorid (AC) from Merck-Schuchardt, G.F.R.

With some compounds, a clear zone was produced around the disk where the agent's concentration was the highest owing to the complete inhibition of bacterial growth. In this case a saturated solution was prepared by dissolving the toxic compounds in N, N-dimethylformamide (DMF, Reanal, Budapest, Hungary). An amount of 25 μ l of these 2–5–10-fold dilutions was dropped onto the disk.

Results

The effects of 30 pesticides (technical grades) on back-mutation of *hcr*⁺ and *hcr*[−] derivatives of *E. coli* WP2 *try*[−] bacteria are shown in Table I.

Three agents proved markedly mutagenic, one of them seemed doubtful and 26 compounds failed to induce reverse mutations.

Orthocide and Unifos were mutagenic for both *hcr*⁺ and *hcr*[−] strains, whereas Ortho-Phaltan was detectably mutagenic only for the *hcr*[−] strain. The mutagenic effect of Tedion was variable; this could however be observed in the *hcr*[−] strain only. In certain experiments this agent formed a ring consisting of a score of colonies (30–40) although this was not consistently reproducible.

The alkylating agents (MNNG, NMUr) and one of the intercalating compounds (AC) showed strong mutagenic effects in both the *hcr*⁺ and *hcr*[−] strains.

The organic solvent (DMF) did not increase the number of revertants above the standard control level.

Novenda, Sumithion, Vapam, Anthio, Unifos, Granosan and Gomex formed a clear zone around the disk when inhibitory concentrations of these compounds were being tested. To prevent this toxic effect, before dropping on the disk, dilutions were made from the agents (see in Materials and methods).

Table I

Effect of pesticides on the reversion to prototrophy of E. coli WP2 try⁻ her⁺ and try⁻ her⁻

Trade name and synonyms	Chemical composition	Reverse	
		her ⁺	her ⁻
Unifos 50 EC Dichlorvos Vapona	Dimethyl-2,2-dichlorovinyl-phosphate (50%)	+	++
Dimecron 50 Phosphamidon	Dimethyl-(2-chloro-2-diethylcarbamoyl-1-methylvinyl)-phosphate (50%)	—	—
Gardona 50 WP	2-Chloro-1-(2,4,5-trichlorophenyl)-vinyl-dimethyl-phosphate (50%)	—	—
Sumithion 50 EC	O,O-Dimethyl-O-(3-methyl-4-nitrophenyl)-phosphothioate (50%)	—	—
Anthio 25 EC Formothion	O,O-Dimethyl-S-(N-methyl-N-formyl-carbamoylmethyl)-phosphothioate (25%)	—	—
Lebaycid 40 WP Fenthion	O,O-Dimethyl-O-(3-methyl-4-thiomethylphenyl)-phosphothioate (40%)	—	—
Phosphothion Malathion	O,O-Dimethyl-S-(1,2-dicarbathoxyethyl)-phosphodithioate (50%)	—	—
Basudin 5 G Diazinon Alfa-tox	O,O-Diethyl-O-6-(2-isopropyl-4-methylpyrimidinyl)-phosphothioate (5%)	—	—
Vapam Metham	Methyldithiocarbamate sodium (33%)	—	—
Sevin 85 WP Carbaryl	1-Naphthyl-N-methylcarbamate (85%)	—	—
Dithane M-45	Manganese-zinc-ethylene-bis-dithiocarbamate (80%)	—	—
Zineb Parazate	Zinc-ethylene-bis-dithiocarbamate (80%)	—	—
Bercema-ferbam 50	Ferric-tris-dimethyl-dithiocarbamate (50%)	—	—
Orthocide 50 WP Captan	N-Trichloromethylthio-4-cyclohexene-1,2-dicarboximide or N-Trichloromethylthio-1,2,3,6-tetrahydro-phthalimide (50%)	+++	+++
Ortho-Phaltan Folpet	N-Trichloromethylthio-phthalamide (50%)	—	++
Tenoran Chloroxuron	N-P-(p-Chlorophenocyphenyl)-N', N'-dimethylcarbamide (50%)	—	—
Aresin	N-(4-Chlorophenyl)-N'-methoxy-N'-methylcarbamide (50%)	—	—
Aktikon	2-Chloro-4-isopropylamino-6-ethyl-s-triazine (90%)	—	—
Buvinol	2-Chloro-4-isopropylamino-6-ethyl-s-triazine (25%) and 2,4,5-Trichlorophenoxy-ethanol (25%)	—	—

Table I (continued)

Trade name and synonyms	Chemical composition	Reverse	
		<i>hcr</i> ⁺	<i>hcr</i> ⁻
<i>Cosan</i>	Sulphur (75%)	—	—
<i>Granosan</i>	Ethylmercuric chloride (2.1% Hg)	—	—
<i>Pol-Thiuram</i>	Tetramethyl-thiuram-disulphide (85%)	—	—
<i>Karathane FN-57</i> Dinocap Arathane Mildex	2-(1-Methylheptyl)-4,6-dinitrophenylcrotonater (25%)	—	—
<i>Hungaria L 10</i> Lindane Gamma BHC	1,2,3,4,5,6-Hexachlorocyclohexane (10%)	—	—
<i>Novenda</i>	4,6-Dinitro- <i>o</i> -cresol (25%)	—	—
<i>Tedion V-18</i> Tetradifon	2,4,4',5-Tetrachlorodiphenyl-sulphone (8%)	—	±
<i>Dymid 80 WP</i> Diphenamid	N,N-Dimethyl-2,2-diphenyl-acetamide (80%)	—	—
<i>Ditrifon 50 WP</i> Trichlorfon Chlorphos	O,O-Dimethyl-(1-hydroxy-2,2,2-trichloro-ethyl) phosphonate (50%)	—	—
<i>Gomex</i>	1,1-Dimethyl-4,4'-bipyridinium-bis-dimethyl-sulphate (28%)	—	—
<i>Dikonirt D</i> Verton D 2,4-D	2,4-Dichlorophenoxy-acetic acid, sodium salt (80%)	—	—
<i>Solvents</i>			
—	N,N-Dimethylformamide	—	—
—	Dist. water	—	—
<i>Positive control</i>			
MNNG	N-Methyl-N'-nitro-N-nitrosoguanidine	+++	+++
NMUr*	N-Nitroso-N-methylurethane	+++	+++
AC	Acridinium chloride	+++	+++

Mutagenic activity, corresponding to the standard: — control; ± doubtful; + weak (20—30 more colonies than the standard control); ++ medium (60—80 more colonies than the standard control); +++ strong (a ring of countless colonies around the disk)

* NMUr being a volatile compound, its mutagenicity did not appear in a ring form around the disk but the number of revertant colonies was increasing on the entire surface of the plate

Discussion

Both the *hcr*⁺ (excision repair competent) and the *hcr*⁻ (excision repair deficient) derivatives of *E. coli* WP2 *try*⁻ strain have been used in an effort to determine whether or not the mutagenic activity of any pesticide was dependent on excision repair. Among the four pesticides found to be mutagenic in the present test, the mutagenicity of Orthocide was independent of the

ability of bacterial repair system. It was markedly mutagenic for both the *hcr*⁺ and *hcr*⁻ strains. The same applied to Unifos to a lesser extent. Ortho-Phaltan and Tedion proved mutagenic only in the repair deficient strain.

FICSOR and NII [10] were the first to show that Captan (as the active constituent of the fungicide Orthocide) was mutagenic to bacteria. CLARKE [11] found an increased number of revertants of *E. coli* WP2 strain when applying fungicides containing three captans.

The insecticide Unifos (the active ingredient of this pesticide is dimethyl-2,2-dicholovinyl-phosphate, DDVP) was mutagenic causing an approximately two-fold increase in the number of revertants in the *hcr*⁺ strain and an approximately four-fold increase in the *hcr*⁻ strain.

DDVP has been shown to alkylate DNA [12] but there are conflicting reports as to its mutagenicity. In bacteria, LÖFROTH *et al.* [13] reported on the induction of streptomycin-independent revertants in a streptomycin-dependent strain of *E. coli* B. Recently, induction of mutations to prototrophy was demonstrated for *E. coli* WP2 *try*⁻ using a semi-quantitative spot test technique by ASHWOOD-SMITH *et al.* [14]. By the same system, DEAN [15] obtained a negative result although mutagenesis was observed in parallel tests with *Serratia marcescens*. Weak positive results in a number of species have been reported by VOOGD *et al.* [16].

The variability of the mutagenic effect of DDVP is supported by the data of BRIDGES *et al.* [17] according to which, in contrast to our own findings, the *uvrA* gene did not affect the mutagenic responses.

In our bacterial test system, the fungicide Ortho-Phaltan (containing thiophthalimide as the active ingredient) is capable of inducing mutation only in the *hcr*⁻ strain, although Ortho-Phaltan chemically differs from Captan insofar that it has a benzene ring while the ring of the latter contains only one unsaturated bond.

The mutagenic activity of fungicides containing three different phthalimide derivatives, has recently been shown in another bacterial test system (*Salmonella typhi-murium* LT2) by SEILER [18].

The three markedly mutagenic agents (Orthocide, Ortho-Phaltan, Unifos) and the one of doubtful effect (Tedion) are extensively used in agriculture and gardening. Although DDVP proved non-mutagenic for mammalian systems [19, 20], it seems of interest to extend investigations on the mutagenicity of the above substances to higher organisms such as *Drosophila* and the mouse.

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NEW SEROTYPES OF *MORGANELLA MORGANII**

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Summary. On the basis of 8 new O and 11 new H antigens determined in 22 strains, the *Morganella morganii* antigenic schema was supplemented with 8 serogroups (O35–O42) and 13 serotypes. Four strains belonged to O groups described earlier and 2 strains contained new O antigens in combination with known O antigens. Known H antigens were present in one strain as a single factor and in one strain as combination of two factors. New H antigens were demonstrated in 5 serotypes in combination with known H antigens. Six out of the 22 isolates were classified into O group 35. Two isolates contained different B-type surface antigens; these factors were not related to *Escherichia coli* B antigens and, unlike the latter, their living suspension gave a higher titre agglutination in OK serum as compared to the boiled culture.

The serology of *Morganella morganii* was first studied by RAUSS, who in 1936 classified 48 strains into 17 serological groups and 7 types [1]. In 1959, RAUSS and VÖRÖS, based on the examination of 222 isolates, elaborated an antigenic schema [2]. After determining 5 further serotypes [3], in 1967 the schema contained 34 O groups and 62 serotypes.

In the Institute of Microbiology, Šafarik University, Košice, from faecal samples of infants' and adults' enteritis, 43 *M. morganii* strains were isolated, 21 of which corresponded to serotypes of the RAUSS–VÖRÖS schema. The remaining 22 strains seemed to differ in antigenic structure from the described serotypes and were, therefore, subjected to further studies.

Materials and methods

Biochemical examinations were performed as described in reference [4].

Serological examinations were made according to KAUFFMANN's method [5]. O antigens were prepared by boiling the cultures for 2 1/2 hr. The O antigens were determined first by slide agglutination in polyvalent typing sera. If a culture failed to agglutinate in these sera, an O serum was prepared against it. To exclude unilateral relationships, all type strains were examined in the new O serum. Then other untypable *M. morganii* strains were examined in the new serum by slide and tube agglutination and by absorption test. Antigenic analysis

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of strains containing complex antigens is demonstrated in separate tables, while strains characterized by single factors are shown in Table VI summarizing the results.

For the preparation of H antigens, broth was seeded from swarm agar plates and the cultures were preserved with 0.5% formalin. Surface antigens were examined in sera prepared with alcoholized suspensions.

Results

Biochemical reactions. All strains produced acid and gas in glucose and acid in galactose and laevulose. Lactose, mannitol, raffinose, sorbitol, sorbose and sucrose were not fermented in 7 days. In NÓGRÁDY's polytropic medium [6], which corresponds to TSI agar except that it contains urea in addition, no H_2S production was recorded. All strains were methyl red positive, Voges-Proskauer negative, failed to utilize citrate in Simmons' medium, hydrolyzed urea, grew in KCN medium, produced no gelatinase in 3% gelatin agar and formed indole; of the amino acid tests only ornithine decarboxylase was positive; phenylalanine was deaminated. On blood agar, 6 out of the 22 strains produced beta-haemolysis. On the basis of these reactions all strains were classified into biotype 1 [2].

Serological studies. Analysis of the O antigens of strains K118, K81, K16 and K17 is shown in Table I. The sign "K" indicates the origin (Košice) of the strains. In O serum the living suspensions agglutinated at higher titres than the corresponding boiled suspensions; the increased agglutinability of living suspensions, attributed to a thermolabile factor, is characteristic of the tribus *Proteus* [1, 7]. Three strains, K118, K81, K16, belonged to the

Table I
Analysis of the O antigens of strains K118, K16, K81 and K17

Strains	O serum K118			O serum K17	
	Unabsorbed	Absorbed by K16 100°C	Absorbed by K17 100°C	Unabsorbed	Absorbed by K118 100°C
K118 living	2560	—	2560	1280	—
K118 100°C	640	—	640	320	—
K16 living	2560	—	2560	320	—
K16 100°C	1280	—	1280	320	—
K81 living	2560	—	2560	320	—
K81 100°C	1280	—	1280	320	—
K17 living	640	—	—	1280	1280
K17 100°C	640	—	—	640	640
Antigenic structure	O36a,b	—	O36b	O36a,c	O36c

same serogroup: absorption of the serum with one of them removed agglutinins for the others. Strain K17 deserves special interest: it agglutinated in lower titre than the others in, and failed to remove all agglutinins from, serum K118. *Vice versa*, after absorption of serum K17 with K118, agglutinins only for the homologous strain were left. It may be concluded that this group of strains is characterized by a poorly developed common antigen ("a"); strains K118, K81 and K16 share the same antigen, that differs from the specific antigen of K17. Thus the antigenic structures can be expressed as O36a,b for the former strains and as O36a,c for K17.

Table II demonstrates strains with the new antigen O37. The presence of a thermolabile antigen in strains K152, K33 and K69 was shown by the fact that in O serum prepared with boiled cultures they practically failed to agglutinate, while in OK serum they reacted well. The identity of O antigens was evident from the complete absorption of the O serum by boiled culture K33. The heated suspension of K33 too absorbed OK serum completely; this finding indicated that the agglutinin-binding capacity had remained intact, *i.e.* the strain contained a B-type surface antigen. This antigen was

Table II

O antigens of strains K152, K33 and K69

Strains	Sera			
	O serum K152 unabsorbed	O serum K152 absorbed by K33 100°C	OK serum K152 unabsorbed	OK serum K152 absorbed by K33 100°C
K152 living	40	—	1280	—
K152 100°C	320	—	640	—
K33 living	20	—	1280	—
K33 100°C	320	—	640	—
K69 living	20	—	1280	—
K69 100°C	320	—	320	—
Antigenic structure	O37	—	O37 : K3	—

designated as K3. In one respect, however, this factor was different from the classical B antigens, as in OK serum prepared with alcoholized suspension, the living culture agglutinated at a higher titre than the boiled one. RAUSS and VÖRÖS [3] described a similar surface antigen: this factor was serologically identical with *Escherichia coli* O112 : B11 antigen, but the living strain agglutinated in O serum at higher titre than the killed one. In *M. morganii* serotype O34:H25 RALOVICH and VÖRÖS [8] demonstrated a similar thermolabile factor related to *E. coli* B4(K58) antigen. In the present study in addi-

tion to antigen K3, another new surface antigen, termed K4, was revealed (strain K1 = O40:K4). Neither antigen K3 nor antigen K4 was related serologically to *E. coli* B antigens.

In addition to antigens O36 and O37 described above, 6 new O antigens were found: O35 (strains K6, K135, K138, K145, K147, K151), O38 (K134), O39 (K168), O40 (K1), O41 (K141) and O42 (K59).

Relationship of the new O antigens to O antigens described earlier is shown in Table III. Strain K14 reacted at a low titre in serum O13, yet it

Table III
O antigens of strain K14

Strains	Serum O13*		Serum K14	
	Unabsorbed	Absorbed by K14 100°C	Unabsorbed	Absorbed by O13* 100°C
O13* living	640	—	640	—
O13* 100°C	640	—	1280	—
K14 living	160	—	5120	2560
K14 100°C	80	—	5120	5120
Antigenic structure	O13a	—	O13a,b	O13b

* Type strain

removed all agglutinins from this serum. Whereas the type strain O13 failed to absorb the agglutinin for strain K14. Accordingly, the strain contains a factor in addition to the common one (type strain O13=O13a, strain K14=O13a, b). An ab-ac relationship was demonstrated between type strain O19 (O19a, b) and strain K64 (O19a, c).

Determination of H antigens. Sera prepared with strains not agglutinating in polyvalent H sera were examined first with slide then with tube agglutination. Part of the strains gave cross-agglutinations.

As shown in Table IV, six strains (K6, K135, K138, K145, K147, K151) contained two H antigens (H26 and H27). In strain K168, antigen H26 was present in combination with a different factor (H32). Further new H antigens were shown in strains K141 (H33) and K59 (H34).

Relationship of the new H antigens to those described earlier. The new H antigens were related to several known H antigens. As shown in Table V, strains K2 and K72 agglutinated in type sera H7 and H24, but the suspension of type strain H24 failed to react in serum H7. After the absorption of serum K72 with antigen H7, the serum still agglutinated K72 (H24). Similarly, when absorbed with antigens H24, serum K72 still contained agglutinins (H35). Accordingly, the antigenic structure of strains K2 and K72 can

Table IV

H antigens of strains K6, K135, K138, K145, K147, K151 and K168

Strains (H antigen)	Serum K6			Serum K168	
	Unabsorbed	Absorbed by K147	Absorbed by K168	Unabsorbed	Absorbed by K6
K6	6400	—	800	3 200	—
K135	6400	—	800	3 200	—
K138	6400	—	400	3 200	—
K145	6400	—	1600	3 200	—
K147	3200	—	800	3 200	—
K151	6400	—	1600	3 200	—
K168	6400	—	—	25 600	1600
Antigenic structure	26,27	—	27	26,32	32

be defined as H7,24. Type antigen H24, which had been regarded as a single factor, was divided into two components (H24, 35).

Strains K69 and K152 (O37) were also related to type antigen H7: in addition, they contained a new factor (H7,28). Strain K33, also belonging to group O37, contained antigen H7 in a different combination (H7,29); the latter factor was present also in strain K134 (O38:H7,29). The H antigen of strain K1 was identical with type antigen H2. Three strains belonging to O group 36 (K16, K118 and K81) contained a new factor (H30) in addition to type antigen H16. In strain K17 of the same O group, however, a different H antigen, (H31) was demonstrated. Strain K14 was characterized by a new antigen, H36, in combination with type antigen H2, while strain K64 (O19a, c) only by H2.

Results are summarized in Table VI.

Table V

H antigens of strains H7, H24, K2 and K72

Strains (H antigen)	Serum H7		Serum K72				Serum H24	
	Un- absorb- ed	Ab- sorbed by K72	Ab- sorbed by H7	Ab- sorbed by K2	Unab- sorbed	Ab- sorbed by H24	Ab- sorbed by K72	Unab- sorbed
H7	12 800	—	—	—	6 400	6 400	—	—
H24	—	—	800	—	400	—	800	3200
K2	12 800	—	400	—	12 800	12 800	—	400
K72	6 400	—	800	—	12 800	12 800	—	3200
Antigenic structure	7	—	24	—	7,24	7	35	24,35

Table VI
Antigenic structure of the examined strains

Designation of strain	Group	Antigens		No. of strains
		O	H	
K14	13	13a,b	2,36	1
K64	19	19a,c	2	1
K2	33	33	7,24	2
K72				
K6	35	35	26,27	6
K135				
K138				
K145				
K147				
K151				
K16	36	36a,b	16,30	3
K81				
K118				
K17	37	36a,c	31	1
K69		37:K3*	7,28	2
K152			7,28	
K33			7,29	1
K134	38	38	7,29	1
K168	39	39	26,32	1
K1	40	40:K4*	2	1
K141	41	41	33	1
K59	42	42	34	1

* Thermolabile antigens

Discussion

Antigenic features of the new isolates were characteristic of the species *M. morganii*. The probable existence of new serotypes has been pointed out earlier. The first antigenic schema [2] containing O groups 1-29, was later

supplemented with O groups 30–34 [3]. The 8 new serogroups and 13 new serotypes described in the present paper increased the number of serogroups from 34 to 42, and the number of serotypes from 62 to 75. In addition, antigens representing groups O13 and O19 were shown to be complex factors: O13a:H10,13 (type strain for O13), O13a,b:H2,36 (strain K14), O19a,b:H5 (type strain for O19) and O19a,c:H2 (strain K64).

H antigens known from our earlier papers were present in 3 types as single factors and in 5 types in combination with new H antigens. Antigen H24 was also a complex (H24,35).

The present study confirmed that B-type thermolabile antigens may occur in *M. morganii*. The two new antigens of this kind demonstrated in the present study were not related to *E. coli* B antigens. Another thermolabile factor was assumed to be responsible for the finding that boiled cultures agglutinated at a lower titre in O sera than living ones.

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INTERFERON INDUCTION AND ACTION IN TRANSFORMED POIKILOTHERMIC CELLS

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Summary. The production and effect of interferon in the virus-transformed cell line TGK₁, originating from kidney cells of *Testudo graeca* were studied and compared to those in the primary cell culture. West Nile virus and Newcastle disease virus were used as inducers. Interferon production in TGK₁ cells began 6 hr later than in the primary cell culture and reached the maximum 64 IU, 18 hr after virus inoculation. In the primary culture, interferon production increased till the 48th hr reaching a fourfold level (256 IU). A significant reduction of the antiviral effect of interferon against vesicular stomatitis virus but not against vaccinia virus was observed in the transformed cells. The decreased interferon production and effect in TGK₁ cells is regarded as a consequence of the disturbance of the interferon regulatory mechanism taking place as a result of the virus-induced transformation.

As reported earlier, infection of primary cell cultures of the tortoise *Testudo graeca* with West Nile virus (WNV) and Semliki Forest virus (SFV) leads to interferon production [1, 2]. In addition the antiviral effect of interferon, synthesis of a stimulator of interferon production as well as of an inhibitor [3], e.g. a completely functioning interferon, was found in these cells.

Our laboratory has recently obtained a virus-transformed poikilothermic cell line TGK₁, originating from kidney cells of *Testudo graeca*. The transformation occurred as a result of adenovirus type 22 inoculation [4].

It was of interest to follow the effect of transformation caused by an adenovirus on the function of the interferon system. Comparative studies on the induction and action of interferon in the primary (TGK) and its descending transformed (TGK₁) cell cultures were therefore carried out.

Materials and methods

Cells. The experiments were performed using monolayer cultures of the transformed cell line TGK₁ [4] grown in medium MEM Eagle Difco containing 0.5% lactalbumin hydrolysate, 10% heated calf serum, 100 U/ml each of penicillin and streptomycin, and adjusted to pH 7.2, as well as monolayer primary cultures of tortoise kidney (TGK) prepared according to SHINDAROV [5] and grown in a medium containing 0.5% lactalbumin hydrolysate, 15% calf serum and antibiotics in saline [5].

Viruses. The viruses used as interferon inducers were, WNV, strain Egypt 101, maintained by intracerebral passages in mice; and NDV strain H, cultivated by intraallantoic passages in chick embryos and applied as an UV-inactivated allantoic fluid [6]. The antiviral

effect of interferon was tested against VSV Indiana strain, having undergone serial passages in L cells and TGK cells, and against vaccinia virus passaged in TGK cells.

Interferon induction. Seven-day monolayer cultures of TGK₁ or TGK, having been grown on the wall of 100 ml Povitzky flasks, were inoculated with 2 ml of 10% brain suspension of WNV in saline, the multiplicity of infection being 2 LD₅₀/cell; with adsorption at room temperature for one hour. Inoculation of the cultures with UV-inactivated NDV was carried out with 2 ml undiluted allantoic fluid (infectious titre of the non-inactivated virus, 10⁵ EID₅₀ per 0.1 ml); with adsorption at room temperature for one hour. After inoculation, the cells were washed twice and cultivated in a maintenance medium of 0.2% lactalbumin hydrolysate in MEM Eagle Difco containing antibiotics and adjusted to pH 7.2. After incubation at 37°C, two flasks per sample were collected and their culture medium was treated as described by LAMPSON *et al.* [7].

Interferon assay. The interferon titre was determined by the CPE inhibition method. For this purpose 4-day-old monolayer test-tube cultures of TGK, pretreated for 24 hours with serial two-fold dilutions of the interferon samples, were infected with 100 TCID₅₀ VSV [1]. The interferon titre was read after incubation at 37°C for 48 hr and was expressed in interferon units (IU) per ml.

Testing the antiviral effect of interferon. Three-day-old monolayer test-tube cultures of TGK₁ resp. TGK cells were treated for 24 hr at 37°C with 100 U interferon obtained from TGK cells inoculated by WNV. After two washings with saline, the cultures were infected with VSV or vaccinia virus, the multiplicity of infection being 1.0 in order to obtain a one step growth cycle. The size of the inoculum was 0.2 ml and the time of adsorption at room temperature, 2 hr. The maintenance medium consisted of MEM Eagle Difco with 0.5% lactalbumin hydrolysate and 5% calf serum. Cultures not treated with interferon served as controls. At the end of the one-step cycle, 4 test tubes per sample were frozen for determination of the virus yield, and the inhibitory effect of interferon.

Virus titration. The infectious titre of the viruses was determined by the end-point dilution method by infecting test-tube cultures of the respective kind, and was estimated according to REED and MUENCH [8].

Results

Induction of interferon in TGK₁ and TGK cells. In order to follow the kinetics of interferon production, samples of the transformed and original cell cultures, inoculated at the same time with WNV or NDV, were collected at 6, 16, 18, 24, 48 and 72 hr. The results obtained are illustrated in Figs 1 and 2.

Fig. 1 shows that the interferon production in transformed TGK₁ cells infected with WNV began after the 12th hr, *e.g.* 6 hr later than in the primary cell culture, and reached its maximum (64 IU/ml) in the 18th hr. This level remained constant till the end of the observation period, whereas in the primary culture, interferon production increased till the 48th hr, reaching the fourfold titre of 256 IU/ml.

The curve of interferon production in cells inoculated with UV-inactivated NDV (Fig. 2) was similar. In this case interferon production in TGK₁ cells was recorded in the 18th hr and reached its maximum of 64 IU/ml in the 24th hr. This level remained constant till the end of the experimental period and was four times lower than that in the primary cell culture.

The antiviral effect of interferon was tested against VSV, known as a standard detector-virus as well as against vaccinia virus.

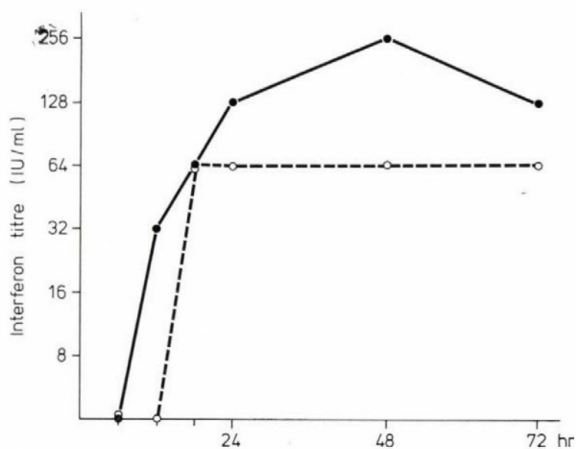


Fig. 1. Interferon production in TGK cells (●—●) and in TGk₁ cell line (○—○) inoculated by WNV

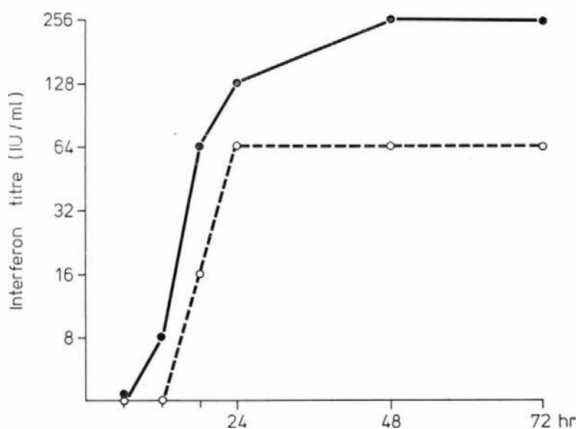


Fig. 2. Interferon production in TGK cells (●—●) and in TGk₁ cell line (○—○) inoculated by UV-inactivated NDV

First the duration of the one-step growth cycle was determined for both viruses in both cell cultures (multiplicity of infection 1.0) (Fig. 3). No significant difference was found in the duration of the cycle including the latent period, and in the character of the virus growth curves in the transformed and normal cells. In the case of VSV, the latent period ended in the 5th hr, so the duration of the cycle was 12 hr, whereas in the case of vaccinia virus, the latent period ended in the 8th hr, thus the duration of the cycle was 15 hr.

In the basic experiments for determining the antiviral effect of interferon, the cell cultures were treated for 24 hr with 100 IU/ml tortoise interferon and then inoculated with virus (multiplicity as indicated above). The

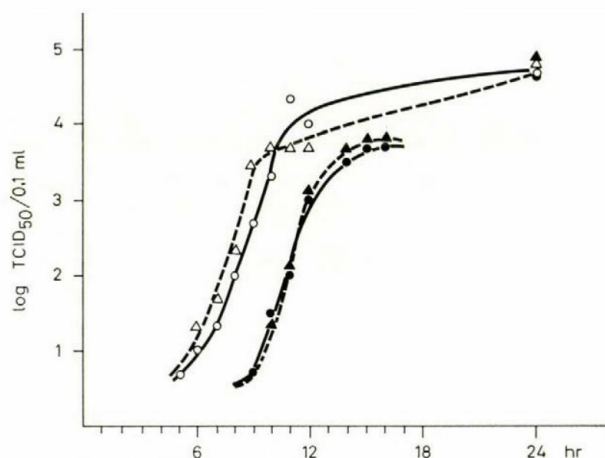


Fig. 3. One-step growth cycle curve of VSV in TGK cells (○—○), and in TGk₁ cell line (△—△) and vaccinia virus in TGK (●—●) and in TGk₁ cells (▲—▲)

Table I

Inhibitory effect of interferon on VSV and vaccinia virus growth in primary cell cultures of Testudo graeca kidney (TGK) and in virus-transformed TGk₁ cell line.

Virus	Cell culture	Virus yield in one-step growth cycle (log TCID ₅₀ /0.1 ml)		Inhibitory effect (Δ log)
		Pretreatment with interferon (100 IU/ml)	Control	
Vesicular stomatitis virus	primary cell culture (TGK)	2.00	4.00	2.00
	TGk ₁ cell line	2.66	4.00	1.33
Vaccinia virus	primary cell culture (TGK)	2.50	3.66	1.16
	TGk ₁ cell line	2.66	4.00	1.33

virus yields at the end of the one-step cycle in the treated and untreated TGk₁ and TGK cells are presented in Table I.

Comparison of the inhibitory effect of interferon in relation to VSV in TGK and TGk₁ cells showed that virus multiplication was inhibited more in the primary TGK culture (2 log) than in TGk₁ cells (1.33 log).

In relation to vaccinia virus, no significant difference was observed in antiviral effects between transformed and normal cultures (inhibitory effect was about 1.2–1.3 log).

Thus, VSV growing in a normal cell culture is more sensitive to interferon than the vaccinia virus in the same culture (2 log and 1.16 log, respec-

tively), whereas in the transformed cell culture no such difference was observed (identical effect, 1.1–1.3 log). Evidently, the transformation had no influence on the antiviral effect of interferon against vaccinia virus but significantly weakened the effect of interferon on the multiplication of VSV.

Discussion

The data in the literature are contradictory concerning the functioning of the interferon system in cell cultures transformed under the action of DNA viruses. A number of workers found no difference in interferon production and effect between DNA virus-transformed cells and normal cells, but they neglected to carry out direct measurements and their conclusions are based on indirect evidence. Thus, STROHL *et al.* [9] found in hamster cell cultures transformed by adenovirus 12 and in normal hamster cell cultures an equal sensitivity to VSV and at the same time a higher resistance of the transformed cells to the lytic adenovirus type 2. No connection between the resistance to repeated infection and sensitivity to interferon was found in polyoma virus-transformed cells [10–12]. OXMAN and BARON [13] and FREEMAN *et al.* [14] did not find differences in interferon sensitivity of SV₄₀-transformed and normal mouse and rat cells.

In contradiction, BRAILOVSKY *et al.* [15] observed a higher sensitivity to VSV in rat, mouse, and hamster cells transformed under the influence of SV₄₀ than in the normal cells of these animals. They explained this finding with an about four times reduced production and antiviral effect of interferon or, generally speaking, with a decreased sensitivity of the interferon system, an early consequence of the transformation.

Our data obtained with adenovirus type 22-transformed cells are similar to those of BRAILOVSKY *et al.* with SV₄₀-transformed rat cells [15], in that they revealed a fourfold decrease of interferon production, a slower interferon production, and a fourfold lowering to 0.66 log of the antiviral effect of interferon against VSV.

The phenomenon observed in transformed kidney cells of *Testudo graeca*, is considered to have resulted from a disturbance of the interferon regulatory mechanism taking place after the virus-induced transformation.

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ABSTRACT OF PAPERS

Virology

INDIRECT HAEMAGGLUTINATION METHOD FOR RAPID DEMONSTRATION OF ANTIBODIES TO RS VIRUS

GY. SZÜCS, M. KENDE and MARIA ÚJ

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Indirect haemagglutination (IHA) test was performed with tanninized sheep erythrocytes sensitized by an antigen obtained from sonicated RS-virus-infected HeLa cells. The same antigen was employed in the complement fixation (CF) test. One hundred human sera were tested with both reactions. Titres up to 1:16 were more frequent with the CF test, whereas over 1:32 the IHA test appeared to be more sensitive (number of positive sera, 28 vs. 14). Fifteen sera had IHA titres 1:128. The titres obtained with the two tests were considerably different for nearly 20% of the sera. It seems reasonable to examine these differences as a function of the time interval between the onset of illness and blood sampling. It is assumed that the antibodies demonstrable by the IHA test are different from CF antibodies.

LOCALIZATION OF ADENO- AND HERPESVIRUSES IN EXPERIMENTAL ANIMALS

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The way of spreading and the localization of type 1 adenovirus and *Herpesvirus hominis* were studied in model experiments carried out in rabbits. Spread of the viruses was followed by immunofluorescence and electron microscopy. Both viruses were detectable in circulating lymphocytes soon after inoculation and in the thymus and in the spleen somewhat later. Occasionally, virus antigens could be detected in liver, kidney and lung cells, too. In the spleen of some animals, specific fluorescence was seen even 60 days after adenovirus infection. Electron microscopy revealed particles in lymphoid organs of infected animals.

CHARACTERIZATION OF A BOVINE HERPESVIRUS ISOLATED
FROM CALVES WITH RESPIRATORY DISEASE

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Virus was isolated from the lungs and bronchial lymph nodes of calves died or emergency-slaughtered in large-scale farms during an epizootic characterized by acute upper respiratory tract disease. In bovine testicle cell monolayers the virus caused plaque-like c.p.e. characterized by cell-rounding, but no syncytium formation. In preparations stained with H-E, COWDRY's type A intranuclear inclusions were demonstrated. The isolates could be propagated in various primary and secondary cell cultures of bovine origin, causing c.p.e. Their average titre was $10^{5.5}$ TCID₅₀. They failed to multiply in either primary or permanent cell cultures of swine, chicken, mouse or monkey origin. Based on physico-chemical characteristics and the nature of the DNA, the isolates proved to belong to the *Herpesvirus* group. In serum neutralization tests the isolates gave no cross reaction with Aujeszky's disease virus, bovine herpes mammillitis virus, equine rhinopneumonitis virus and infectious bovine rhinotracheitis virus. They were apathogenic for common laboratory animals. In colostrum-deprived newborn calves inoculated intratracheally, the virus produced high fever, prostration and rhinitis. In accordance with literary data, antibody to the virus was not found in the sera of calves infected either naturally or experimentally.

ISOLATION OF A NON-ONCOGENIC HERPESVIRUS FROM TURKEYS

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A cytopathogenic agent was isolated in duck embryonic fibroblast cell cultures from healthy turkeys. In the affected cells, COWDRY's type A inclusions were seen. The specific c.p.e. could be prevented by the use of a halogenated uridine derivative. The spontaneously released virus passed through the Millipore membrane of 450 nm pore diameter, while the virus obtained from artificially disintegrated cells passed through membranes of 220 nm. Electron micrographs showed in the cell nuclei virus particles 80–100 nm in diameter and hexagonal in cross section. Occasionally, particles of 180–200 nm occurred between the two layers of the nuclear membrane. Intact virus particles rarely occurred in the cytoplasm. The "A" antigen of the isolate is related serologically to the corresponding antigen of Marek's disease (MD) herpes virus. Chickens genetically highly susceptible to MD were inoculated with

large doses of the isolate; all remained healthy, but a cellular reaction reminding of that occurring in MD was observed in some organs. One-day old chickens inoculated with low doses of the isolate proved fully resistant when challenged with virulent MD herpes virus.

FATAL PNEUMONIA IN INFANTS ASSOCIATED WITH ADENOVIRUS
TYPE 7 AND INFLUENZA A2 VIRUSES

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In a hospital ward the majority of infants developed severe bronchopneumonia and 24 of them died. Virus isolation and/or serological results have shown that in 9 of 11 cases adenovirus type 7, and in 2 cases influenza A2 virus was the aetiological agent. Type 7 adenovirus was isolated from the lung tissue and the heart muscle specimens obtained at necropsy of 3 cases.

SUSCEPTIBILITY OF THE DOMESTIC PIG TO HUMAN INFLUENZA
A(H3N2) VIRUS

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A rapidly spreading outbreak was observed in swine stocks in areas affected by a human epidemic caused by influenza A(H3N2) virus. Screening before the outbreaks failed to reveal positive sera, whereas 20–58 days later 52–92% of the pigs in the affected stocks had haemagglutination-inhibiting antibodies to A(H3N2) virus; the antibodies persisted for 6 to 10 months. Experimental infection of pigs resulted in circulation of the virus for at least 5 weeks. Influenza A(H3N2) virus was isolated from the nasal and bronchial mucous membrane of a one-month-old piglet. Antibody production to influenza virus was inhibited by the simultaneous presence of diarrhoea due to *Escherichia coli* infection.

INFECTION OF HORSES WITH EQUINE INFLUENZA A1 AND HUMAN
A(H3N2) VIRUS IN HUNGARY

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Three strains of influenza A1 equi virus were isolated in 1973 from the nasal discharge of horses affected by a mild acute respiratory outbreak. Of 286 serum samples randomly collected from convalescent horses, 57% contained

haemagglutination-inhibiting (HI) antibodies to the same virus in a titre of 1:16 to 1:64. To influenza equi A2 only 18% of the sera, those from 5 year-old or older animals, had HI antibodies, presumably residual ones formed during the 1968 epizootic. Of the tested blood samples 39 (21%) contained HI antibodies to human influenza A(H3N2)Hong Kong(1)68 strain. These antibodies remained demonstrable for 5-8 months; their specificity was supported by the results of the virus neutralization test.

STUDIES ON THE NEUROVIRULENCE OF NEWCASTLE DISEASE VIRUS (NDV) STRAINS

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Avirulent NDV strains are not neurovirulent in chickens. Their most characteristic marker is the lack of plaque formation in chicken embryonic fibroblast cell cultures. As to the interrelation between neurovirulence, plaque formation and virus multiplication the following conclusions have been drawn. (i) Avirulent NDV strains are not neurovirulent because they do not multiply in the neural tissues of the chicken. (ii) Being able to multiply in the brain of the chick embryo, they show neurotropism, but do not kill the chick embryo. (iii) In chicken embryonic fibroblast cultures they multiply less intensely than the more virulent strains and, not being cytopathogenic, they do not form plaques. (iv) In epithelial cell cultures they behave like the more virulent strains, *viz.* they multiply well, cause cytopathogenic lesions and form plaques. (v) Under the effect of Mg^{2+} or DEAE dextran they form plaques even in fibroblastic cultures, due to stimulation of the cytopathogenicity, without enhancing virus multiplication. (vi) For neurovirulence and plaque formation in addition to virus multiplication, an ability of the virus to destroy cells is needed.

ISOLATION OF REOVIRUS TYPE 1 FROM LAMBS EXHIBITING RESPIRATORY AND GASTROENTERIC SYMPTOMS

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A reovirus strain was isolated in secondary cultures of lamb kidney cells, from nasal and faecal swabs taken from lambs affected by an outbreak characterized by respiratory and gastroenteric symptoms. This was the first

isolation of reovirus type 1 from sheep. Cytopathic changes appeared initially 4-5, later 2-3, days after inoculation of cultures. Haematoxylin-eosin-stained preparations showed acidophilic cytoplasmic inclusions. These were minute in size and irregular in shape at the beginning, but coalesced and formed a perinuclear ring subsequently. Electronmicroscopically, spherical virus particles 60-70 nm in diameter and crystal-like in structure were seen in the cytoplasm. Physico-chemical investigations and the type of the viral nucleic acid showed that the isolate was a reovirus agglutinating human erythrocytes. It was closely related to the reovirus type 1 prototype strain as shown by the haemagglutination-inhibition and virus neutralization tests. Virus-neutralizing serum antibodies appeared and soon reached a titre of 1:64-1:128 in naturally infected lambs. Experimentally infected lambs displayed the same symptoms, developed antibodies, and excreted the virus. It is concluded that reovirus type 1 played an aetiological role in the outbreak.

CHARACTERIZATION OF THE VIRUS OF GOOSE INFLUENZA

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A virus strain that had been isolated in embryonated goose eggs from goslings died of goose influenza has been adapted to goose embryonic fibroblast cell cultures. Cells in the infected cultures rounded off, and eosinophilic nuclear inclusions were observed in stained preparations. The virus contained DNA and was resistant to chloroform and heating; H 3 did not influence its infectivity. Electron micrographs prepared by the negative staining technique showed virus particles of cubic symmetry 20-22 nm in diameter. The one-step replication cycle of the virus lasts 96-108 hr, whereafter 10% of the virions remain cell-associated. Replication was vigorous in cell cultures mainly consisting of dividing cells. The virus strain B characterized by the authors is antigenically related the Hungarian isolates "H" and "LB", and the strains isolated from similar outbreaks in the Netherlands, France and in the Soviet Union. It is suggested that the virus of goose influenza is a parvovirus.

STOMATITIS PAPULOSA VIRUS INFECTION IN HUNGARIAN CATTLE STOCKS

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The occurrence in Hungary of papulous stomatitis in calves and young cattle has been shown previously. The virus was isolated in bovine kidney

cell monolayers. Cytopathic changes, *viz.*, reticular cytoplasmic degeneration, cytoplasmic inclusions eosinophilic as well as basophilic granules and nuclear pycnosis, appeared on the 3rd to 5th day after inoculation. Green fluorescence of acridine-orange, characteristic of DNA, was demonstrated in cytoplasmic inclusions. The isolates proved resistant to heat, sensitive to chloroform and did not reproduce in the presence 50 $\mu\text{g/ml}$ of idoxuridine. Thus, they belong to the pox-like (paravaccinia) group of viruses. Thirty-six calves 1–5 months of age were tested for virus-neutralizing antibodies against the homologous virus strain. One serum had a 1:4, another a 1:8 neutralizing antibody titre as tested against 100–1000 TCID₅₀ of virus. Differentiation from virus diarrhoea, bullous stomatitis and foot-and-mouth disease may be difficult, for the early-stage papules in these diseases resemble the papules occurring in papulous stomatitis.

REPEATED CHROMOSOME EXAMINATION OF PATIENTS
IN REMISSION OF SUBACUTE SCLEROSING PANENCEPHALITIS (SSPE)

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In recent years, chromosome breakage or pulverization has been demonstrated in leukocytes of 9 patients with SSPE. Two of these are in remission since several years. When re-examined 4 and 5 years, respectively, after the first examination, the chromosomal abnormalities were found to have increased in frequency, in one case from 10% to 31%, while in the other, slightly. The clinical condition failed to reflect the chromosomal pattern.

POSSIBLE ROLE OF HERPESVIRUS HOMINIS IN DEPRESSIVE ILLNESS

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Thirty-nine patients suffering from endogenous depression were tested for neutralizing antibodies to *Herpesvirus hominis* types 1 and 2 as well as measles virus. The frequency and the titres of *H. hominis* type 1 antibodies were substantially higher in this group than in an age- and sex-matched control group consisting of healthy subjects and of patients with various mental diseases. A similar difference could not be demonstrated with ham-agglutination-inhibiting antibodies to measles virus.

SERUM ANTIBODIES TO MEASLES, MUMPS, INFLUENZA, PARAINFLUENZA,
RUBEOLA AND VACCINIA VIRUSES IN PATIENTS
WITH MULTIPLE SCLEROSIS (MS)

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Serum samples from 90 patients suffering from MS were tested for antibodies to several viruses. Antibodies to measles, rubella and vaccinia viruses were higher in both incidence and titre in the MS group than in a control group. The difference in frequency of anti-vaccinia antibodies was significant at the $P < 0.05$ level.

USE OF IMMUNEADSORBENTS PREPARED WITH GLUTARALDEHYDE
FOR HEPATITIS B ANTIGEN (HB_{Ag}) PURIFICATION

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Anti-HB suitable for labelling with ^{125}I was prepared by means of immuneadsorption. Human serum containing high-titre HB_{Ag} was concentrated by adsorbing it onto $\text{Al}(\text{OH})_3$ gel, successive elution and chromatography on DEAE cellulose. To find the conditions suitable for the concentration with gel-adsorption, the adsorption properties of HB_{Ag} were studied. For this purpose, *ad*, and *ay* subtypes of HB_{Ag}, both purified by density gradient centrifugation were adsorbed onto gel and eluted with 0.005 to 0.32 M phosphate buffer. The elution curves for the two subtypes ran parallel; 50% adsorption equilibrium was found at a molarity of 0.06 M phosphate buffer. Subsequently, the partially purified antigen, was concentrated and adding bovine albumin as carrier, HB_{Ag} containing adsorbent was prepared with glutaraldehyde. To this adsorbent, anti-HB human serum was added and following incubation at 37°C, the adsorbent was washed several times with saline. The fixed antibody was eluted with glycine buffer pH 2.5 and the purified anti-HB thus obtained was labelled with ^{125}I . The labelled antibody was compared with the corresponding Abbot preparation, using HB_{Ag}-positive and HB_{Ag}-negative sera for comparison. Positive and negative sera were well distinguishable by this method. In the second part of the experiments, purified HB_{Ag} was prepared by similar technique.

EFFECTS ON PLANTS OF SERA FROM PATIENTS WITH VIRAL HEPATITIS

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Leaves of *Nicotiana sylvestris*, *N. langsdorfi*, *N. rustica*, *N. clevelandi*, *Chenopodium quinoa*, *Ch. foetidum*, *Zinnia elegans*, cucumber and lettuce were inoculated with blood sera. Forty-two sera of which 23 were HBsAg-positive and 19 HBsAg-negative, were obtained from patients suffering from viral hepatitis, 7 sera from healthy HBsAg-negative subjects; altogether 165 inoculations were performed. Of *N. sylvestris* and *N. langsdorfi* leaves inoculated with HBsAg-positive hepatitis sera 75% showed withering and crosping and, at a later stage, stunting. Necrosis was never observed. Other plants displayed no damage or the inoculations were too few for allowing conclusion. Of the 15 HBsAg-negative hepatitis sera tested on *N. sylvestris* and *N. langsdorfi*, six and two, respectively, caused lesions similar to those seen with HBsAg-positive sera. HBsAg-negative control sera caused no damage. Injection of serum into the leaves was found to be a more suitable procedure than the rubbing-in method.

DYNAMICS OF HEPATITIS B ANTIGEN AND ANTIBODY
COMPLEMENT-FIXATION TITRES IN THE BLOOD SERUM OF PATIENTS
WITH HEPATITIS B

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HBsAg-positive cases (157 males and 173 females) clinically diagnosed as serum hepatitis were tested for HBsAg, anti-HBs and anticomplementary (AC) activity suggestive of immune complexes, at intervals from admission to hospital to 2 years after discharge. One hundred and ninety patients including 76 males and 114 females had neither anti-HBs nor AC activity demonstrable at admission (for sex distribution, $\chi^2 = 8.962$ $P < 0.01$). By the clinical recovery, 114 male (73%) and 59 female patients had developed anti-HBs and/or AC activity ($\chi^2 = 33.30$ $P < 0.001$). The presence of demonstrable anti-HBs or AC in the first serum sample seemed to be favourable prognostically; their subsequent appearance did not modify the course significantly. The dynamics of HBsAg showed a good correlation with course of the illness.

THE FREQUENCY OF HEPATITIS B ANTIGEN (HB_{Ag}) CARRIERS
IN BUDAPESTTERÉZ JOÓ-SZABADOS, ALLA FILONENKO, ERZSÉBET KOVÁCS, CSILLA NÉMETH,
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Inhabitants of Budapest 0–60 years of age were screened for HB_{Ag} by counter-electrophoresis. Five thousand serum samples, 2000 from children and 3000 from adults, were tested. HB_{Ag} could be demonstrated in none of the 1000 newborn sera, though 1% of the mothers were positive. Similarly, no carrier was found among children below 3 years of age. Only one (0.4%) of those between 3 and 10 years proved to be positive. The frequency of carriers was the same for the age group 10 to 17 years, for the blood donors 18 to 30 years of age and for pregnant women, viz., 2.7% for males and 1.1% for females. It tended downwards over 30 and approached 0% between 50 and 60 years of age.

CYTOPATHIC EFFECT OF MYCOPLASMA AND ACHOLEPLASMA SPECIES
IN GOOSE AND CHICKEN EMBRYONIC TISSUE CULTURES

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The cytopathic effect of *Mycoplasma* and *Acholeplasma* strains isolated from one day-old goslings, embryonated goose eggs and goose embryonic fibroblast tissue cultures was studied in goose and chicken embryonic fibroblast tissue cultures. In the cultures of goose origin all the tested strains except *A. laidlawi* caused vacuolation and cytoplasmic inclusions. Similar changes were observed in chicken embryonic fibroblast cultures. In addition, pycnotic changes appeared in cultures infected with *A. laidlawi*. These kinds of cytopathic changes should call attention to contamination of tissue cultures by *Mycoplasma* or *Acholeplasma*, microorganisms often present in embryos used for preparation of tissue cultures.

Tumour viruses, interferon

DEMONSTRATION OF GROUP-SPECIFIC "A" (GS-A) ANTIGEN AND GS-A ANTIBODIES BY RADIOIMMUNOASSAY IN CHICKEN AND JAPANESE QUAIL EMBRYOS

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The material to be tested was obtained from two poultry farms and a breed of Japanese quail. The gs-a antigen was determined by radioimmunoassay in viscera and fibroblast cells and in the nutrient medium of cultured cells. The anti-RSV neutralizing and gs-a antibodies were determined in the yolks. For the chicken embryos, the RIF test was negative; the virus strains failed to form foci in the Japanese quail fibroblast cultures. The positive radioimmunoassay and virus neutralization tests suggested that the hen eggs from both breeds had been contaminated with avian leucosis virus. The criteria for the SPF stock were presented.

IMMUNOGENIC EFFECT OF TUMOUR CELL MEMBRANE AND ITS SUBFRACTIONS IN THE RAUSCHER LEUKAEMIA MODEL

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Cell membrane of 96–97% purity was prepared from Rauscher leukaemia cells of Balb/c mice. The preparation was treated with 1% formalin and injected into Balb/c mice intraperitoneally. Virus-neutralizing and cytotoxic antibodies were formed. The mice given 200 μ g of whole-membrane protein and 100 μ g as a secondary stimulus became fully protected against 10^3 FFU of Rauscher virus; those challenged with 10^4 tumour cells showed a three-fold survival time as compared to the control. By sucrose-gradient centrifugation the leukaemia virions were removed and three subfractions were prepared from the whole membrane. The subfraction of 1.16 sucrose density had no preventive effect. Of each of the subfractions of 1.12 and 1.14 sucrose density 200 μ g membrane protein was administered intraperitoneally. The immunization inhibited the progress of leukaemia induced by 10^3 FFU virus or 10^4 tumour cells. Immunity induced by the 1.14 density subfraction was especially pronounced, leading to an equilibrium between the multiplication and the immunological elimination of the tumour cells.

THE ROLE OF VIRUS MULTIPLICATION AND ANTIGENIC EXPRESSION
IN THE PATHOGENESIS OF RAUSCHER LEUKAEMIA

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The appearance of a significant tumour-specific immune response in mice sensitive to the erythroblastosis virus (SSFV) of the Rauscher virus complex is a function of the resistance to the helper virus (LLV); the duration of remission is determined by the duration of the expression of the virus-induced cell-surface antigens. In Balb/c mice, a strain susceptible to the helper virus, the fast-growing Rauscher virus reaches the immunocompetent cells before the antigenic stimulus and blocks the efferent part of the immune response, especially the precursors of antibody-forming cells. In DBA mice, resistant to the helper virus, exacerbation of leukaemia is caused by the disappearance of the virus-induced cell-surface antigen, a consequence of the masking effect of sialic acid. The loss of the immunogenicity effect of sialic acid. The loss of the immunogenicity and immunosensitivity of the tumour cells results in a rapid progression and metastatisation of the leukaemia. In mice susceptible to SFFV and resistant to LLV, such as DBA/1, DBA/2, (DBA)1xBalb(c)F1, (DBA)1xC57B1(10Sn)F1 mice, a relationship was demonstrated between the length of the remission and the H-2 genotype. The remission-prolonging effect of the H-2^b genotype seems to be active *via* the regulation of the duration of the antigenic expression.

ANTIGENIC STRUCTURE OF THE TRANSPLANTABLE HEPATOMA
LINE ESTABLISHED FROM A PRIMARY TUMOUR INDUCED
BY MC-29 VIRUS

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A transplantable tumour line was established from a liver tumour induced by the MC-29 virus of the avian leucosis group. Rabbits and goats were hyperimmunized with liver homogenates and sera of newly-hatched chickens, with chicken embryo cells and cells obtained from the 23rd-30th subcutaneous passage of the tumour. Soluble antigens of tumour and liver homogenates were compared by gel-diffusion and immunoelectrophoretic analysis, using the antisera. After absorption of the antisera with spleen, lung, kidney, muscle and brain homogenates and serum of chicken, the serum reacted with three liver-specific antigens. These antigens were present in the

tumour tissue as well, but in reduced amounts. The degree of the decrease and the electrophoretic mobility of antigens were estimated, comparing the hepatoma and liver tissue. The results indicated the hepatocellular origin of the transplantable tumour line.

THE EFFECT OF BURSECTOMY AND THYMECTOMY ON THE DEVELOPMENT
OF THE TRANSPLANTABLE HEPATOMA ESTABLISHED
FROM A PRIMARY TUMOUR INDUCED BY MC-29 VIRUS

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Surgical thymectomy and bursectomy were performed on newly-hatched Hunnia hybrid chickens to study cellular (thymus-dependent) immunity and humoral immunity (dependent on the bursa of Fabricius), on development of the transplantable hepatoma established from a primary tumour induced by MC-29 virus. Tumours were transplanted on the day of hatching, and on the 7th and 14th days after hatching. The effect of bursectomy on antibody synthesis was controlled serologically, using SRBC as antigen. Thymectomy and bursectomy had no effect on development of the tumour transplanted on the day of hatching; all the animals died, indicating the immaturity of the surveillance mechanism at this age, which could not be influenced by the surgical interventions in our system. Death rate due to tumours transplanted on the 14th day after hatching was higher in thymectomized chicks than in the controls. This shows the pathogenetic role of cellular immunity in tumour development. The effects of bursectomy on tumour development proved to be inconclusive as it was the case in the experiments of RADZICHOVSKAYA and of McARTHUR *et al.*, who used Rous sarcoma as test tumour. Four mg (semi-dry weight) living *Mycobacterium bovis* (BCG) or *Mycobacterium* W-115 given together with the tumour tissue significantly diminished the incidence of death due to tumours, and the chicks with a positive tumour take showed a markedly slower progression of the disease.

INFECTION OF CHRONIC LYMPHOID LEUKAEMIA (CLL) LYMPHOCYTES
WITH THE EPSTEIN-BARR VIRUS IN VITRO

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Fourteen patients with CLL were examined. Antibody to the Epstein-Barr virus (EBV) was demonstrable in each of them, in 4 cases in high titres; the lymphocytes from the patients were not or only slightly stimulated by

UV-inactivated EBV. The lack of immunological stimulus pointed to a deficiency of cell-mediated immunity. In 2-3% of CLL lymphocytes, the synthesis of EBV-associated nuclear antigen (EBVNA), early sign of virus-cell interaction, was observed on the 10th day following EBV infection *in vitro*. In acridine-orange-stained preparations of EBV-infected cells the presence of transformed cells was demonstrated. It is suggested that the CLL cells are susceptible to EBV infection *in vitro*, but, presumably owing to the high-level EBV antibodies, they are never infected *in vivo* (CLL cells do not contain the EBV genome).

EPSTEIN-BARR VIRUS (EBV) ANTIBODIES IN INFECTIOUS MONONUCLEOSIS (IM)

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Serum samples from 116 patients with IM were examined for anti-VCA (IgG), anti-EBNA and EBV-specific IgM antibodies. EBV-specific IgM appeared in the incubation period, to disappear several weeks after the acute phase. Anti-VCA appeared on the 14th day or somewhat later, whereas the antibody to the EBV-associated nuclear antigen (anti-EBNA) could be demonstrated only after 5-6 months. The predominance of EBV antibodies is especially characteristic of Paul-Bunnell-negative cases. Fresh EBV infection is characterized by the presence of anti-VCA and EBV-specific IgM and the absence of anti-EBNA antibodies. The serological diagnosis of EBV infection cannot be based on a single determination of anti-VCA antibody, for this may be present in high titres in old infections, while the titres may be low even in fresh infections. Titration of the three-membrane antibody complex gives reliable results in the majority of IM cases.

EPSTEIN-BARR VIRUS (EBV) ANTIBODIES IN INFECTIOUS MONONUCLEOSIS (IM)

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Serum samples from 33 IM cases were examined for anti-EBV antibodies by the indirect immunofluorescence technique, using cultured cells of Burkitt lymphoma origin (P3J-HRIK and RAJI). The specimens were tested at dilutions 1:10. Antibodies were detected in 21 cases. From 12 cases three

or more blood samples were tested during a half-year period. In two cases all samples were negative, in one case only the 5th and the 7th samples were positive. In one case all the 7 samples contained demonstrable amounts of EBV antibodies. The difficulties in the diagnosis of virus latency and fresh infection, the fluctuation of the antibody level and complexity of the equilibrium between virus, cell and immune activities are discussed.

EFFECTS OF IDOXURIDINE (IDU) AND ARGININE DEPRIVATION
ON cAMP METABOLISM IN HSV-2-TRANSFORMED CELLS

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In hamster embryonic fibroblast cells, the intracellular cAMP level was not influenced by IDU treatment or arginine deprivation. However, a considerable amount of hormone-sensitive (glucagon, epinephrine) adenylyl cyclate was found in the cytosol fraction of the treated cells. The location and specific activity of the hormone-sensitive adenylyl cyclase were also changed in the subfractions of the cytoplasmic membrane of treated cells. The cAMP phosphodiesterase activity of the cells increased, whereas the cAMP dependence and sensitivity to protamine and histone of the membrane-fixed protein kinase was reduced. It is suggested that the cAMP metabolism of HSV-2-transformed cells is only partially similar to the normal cell metabolism.

SYNTHESIS OF INFECTIOUS DNA AND VIRAL PARTICLES
IN TUMOUR CELLS INDUCED BY SIMIAN ADENOVIRUS 7

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Methods of extraction and assay of infectious DNA from BSC-1 cells productively infected with simian adenovirus 7 (SA7) TÁLAS and BUTEL 1974, were used to investigate the activity of viral genome in hamster tumour cells. Tumour cells induced by SA7 were extracted by the Hirt procedure. Viral infectivity was associated with both the crude Hirt pellet and the Hirt supernatant fractions. The activity was sensitive to DNase, phenol and proteolytic enzymes, but resistant to RNase and SA7-specific antiserum. DEAE dextran was required for detecting infectivity. By means of DNA-DNA filter hybridization technique it was demonstrated that the tumour cells contained 2.8–28 copies of the viral genome per cell. Moreover, very small amounts of

free virus (5×10^4 p.f.u./ 10^6 cells) were recovered when large quantities of tumour cells (800 mg) had disrupted and passed in permissive tissue culture. The results based on infectious centre assays and an immunofluorescence technique showed that less than 0.01% of the tumour cells was synthesizing virus spontaneously. The studies indicate differences in the activity of SA7 viral genome in individual tumour cells.

ABORTIVE INFECTION OF MURINE FIBROBLAST CELLS WITH HUMAN CYTOMEGALOVIRUS (HCMV)

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Reproduction of HCMV is considered host-specific. The authors had observed an incomplete reproduction cycle in primary embryonic mouse cells: adsorption, penetration and early cytopathic effect were observed and some virus-specific antigens were developed. Since these antigens appear in the presence of ara-C as well, they are considered to be coded for by parental virus, DNA. The early antigens reach the highest intensity on the second day following inoculation and disappear by the 7th or 8th day. In cell cultures pre-treated with idoxuridine (IDU) the cytopathic effect is more pronounced, the expression of the early antigens is accelerated and the fluorescence declines so slowly that it does not disappear before the 15th–16th day. HCMV-infected mouse cells do not support the reproduction of vesicular stomatitis virus and herpes simplex virus. The interference is not interferon-dependent. HCMV-specific antigens could be induced in IDA-treated and arginine-deprived cells from which these antigens had disappeared.

CELL-SURFACE ANTIGENS INDUCED BY HUMAN CYTOMEGALOVIRUS (HCMV)

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Development of HCMV-induced membrane antigens was studied in permissive human embryonic fibroblasts and in non-permissive systems, *viz.*, mouse embryonic fibroblasts and arginine-deprived human embryonic fibroblasts. Inoculation was followed by the development of cell-surface antigens, which did not absorb virus-neutralizing and fluorescent antibodies from the anti-HCMV antisera. These antigens appeared in both permissive and non-permissive cells and disappeared soon from the cell surface. Inhibitors of DNA

synthesis (IDU, ara-C) did not, those of protein synthesis (puromycin, cycloheximide) did, prevent their formation. Absorption of HCMV antiserum with cells bearing subsequently produced surface antigens substantially reduced the neutralizing and fluorescent antibody titres of the antiserum.

THE INFLUENCE OF INTERFERON ON THE IMMUNE RESPONSE OF CHICKENS

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The inhibition of the humoral and cell-mediated immune response observed in adenovirus-inoculated chickens is attributed to the release of interferon. To obtain further information, the influence of interferon on the immune response of chickens to sheep erythrocytes was examined. A purified and concentrated interferon preparation produced in chicken lymphocytes infected with adenovirus *in vitro*, was used. The interferon was administered intravenously two days before the injection of sheep erythrocytes. Antibody formation in the splenic lymphocytes of the interferon-treated chickens was inhibited. Chicken lymphocytes cultivate *in vitro* in the presence of interferon gave a reduced response to PHA.

COMPARATIVE PHYSICO-CHEMICAL STUDY OF INTERFERONS PRODUCED IN CHICKEN FIBROBLAST CELLS AND LEUKOCYTES

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The interferons obtained from chicken fibroblast cells had molecular weights of 37 000 and 28 000 daltons. The respective values for the interferons obtained from chicken leukocytes were 32 000 and 23 000 daltons. Experiments were performed to demonstrate the dimerous nature of the interferons, but the presence of a monomeric form could not be proved by using either the complex-forming compound EDTA or low ion strength. The iso-electric points of the preparations supports the view that the interferons consist of several components with isoelectric points in an approximately identical pH range.

SEPARATION OF THE ANTIVIRAL AND PRIMING EFFECTS
OF INTERFERON IN L CELLS

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Incubation of L cells for 2 hr with 25 $\mu\text{g/ml}$ and subsequently for 22 hr with 10 $\mu\text{g/ml}$, cycloheximide substantially protracted the development of the antiviral effect of interferon, but did not prevent the development of the interferon's priming effect. The primed statue of the cells ceased in 24 hr after removal of the interferon, whereas their antiviral resistance persisted, apart from a slight decline in the first 14 hr, practically unchanged through the 3-day observation period.

ROLE OF ADENO-ASSOCIATED VIRUS (AAV) IN INTERFERON
INDUCTION BY ADENOVIRUSES

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Fractions of CsCl density gradient prepared from a strain of adenovirus type 12 proved to be contaminated with AAV as shown by electron microscopy. Since the same adenovirus strain had proved to be an outstandingly good interferon inducer, it was supposed that this ability might be related to the presence of the satellite virus. Comparative studies were performed with an AAV-free and an AAV-carrying strain of adenovirus type 12 and an AAV-free strain of adenovirus type 6. It is concluded that the high interferonogenicity of adenovirus type 12 cannot be attributed to the presence of AAV.

Bacteriology

ELECTRON MICROSCOPIC STUDIES ON BACILLUS MEGATERIUM PHAGES

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Megacin A was present in varying amounts in the lysate of 18 *Bacillus megaterium* strains showing total lysis after mitomycin induction. At higher mitomycin concentrations (5 $\mu\text{g/ml}$), the lysates contained 1 to 4 morphologically different phage particles or defective phage structures. The association between phages and megacin A production is analysed.

ELECTRON MICROSCOPIC AND BIOLOGICAL STUDIES
ON A HIGHLY SPECIFIC ANTHRAX PHAGE

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A highly specific phage (AP 50) isolated from soil acted upon strain Sterne CN 18-74 used for its isolation, and on two other *Bacillus anthracis* strains but not upon other *Bacillus* sp. strains. The phage differed from other anthrax phages not only in its host range but also in UV, pH and heat resistance and in antigenic structure. The phage was characterized by a double layer coat on its hexagonal head; it was inactivated by chloroform and ether; thin layer chromatography of its chloroform-methanol extract showed the presence of ethanolamine.

NEW MORGANELLA MORGANII SEROTYPES

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Eight new O and 11 new H antigens belonging to 22 *Morganella morganii* strains were analyzed and the antigenic schema of RAUSS and VÖRÖS has been supplemented with 8 serogroups (O35-O42) and 13 serotypes. Serogroup 36 was divided into two subgroups; 2 out of 4 strains showing a close relationship to known antigens contained new factors and were classified into new subgroups. Twenty-five per cent of the strains belonged to serogroup O35. Some serotypes were characterized by combinations of two antigens. Two different B-type surface antigens, unrelated to *Escherichia coli* B antigens, were demonstrated and another thermolabile factor was determined.

CLASSIFICATION OF PSEUDOMONAS AERUGINOSA O ANTIGENS
BY IMMUNOELECTROPHORESIS

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Heated saline extracts of 89 strains and L₁, LPS, trichloroacetic acid and sodium hydroxide extracts of 23 strains representing O antigens of LÁNYI's *Pseudomonas aeruginosa* antigenic schema were analyzed by immunoelectrophoresis and electrodensitometric measurement of precipitation lines.

The strains were classified into five immunoelectrophoretic groups. Group I: two lines running at different rate towards the anode; subgroup Ia: moderately alkali resistant antigens (O2, O3a,3b, O3c, O3a,3d, O3a,3d,3e, O3d,3f); subgroup Ib: alkali resistant antigens (O1, O6); subgroup Ic: alkali sensitive antigens (O4a,4c, O5a,5b,5c, O5a,5b,5d, O5a,5d, O10a, O10a,10b). Group II: triple line at the starting well, alkali sensitive (O4a,4b, O4a,4d). Group III: triple line at the starting well, alkali resistant; subgroup IIIa: L₁ fraction reactive (O12); subgroup IIIb: L₁ fraction non-reactive (O13). Group IV: triple line on the cathode side, alkali resistant, L₁ fraction non-reactive (O7a,7b, O7a,7c, O11). Group V: single line towards the anode, alkali sensitive, L₁ fraction and trichloroacetic acid extract non-reactive (O8, O9).

SEROLOGICAL STUDIES ON DIFFERENT ANTIGEN EXTRACTS AND FRACTIONS OF *PSEUDOMONAS AERUGINOSA*

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Serological activity of extracts prepared from 23 antigen type strains of *Pseudomonas aeruginosa* was examined by indirect haemagglutination. Fractions of phenol water extracts (crude aqueous phase, L₁ and purified LPS) and trichloroacetic acid extracts gave usually high titre haemagglutination in the homologous serum. Exceptions were as follows. (i) Crude aqueous phase extract of strains O7a,7b, O7a,7c, O11 and O13 and LPS extract of strain O7a,7c gave high titre reactions only after heating at 100°C. (ii) L₁ fractions of strains O7a,7b, O7a,7c, O11 and O13 failed to react either in haemagglutination or in haemagglutination inhibition test. (iii) Unheated and heated crude aqueous phase, L₁, TCA, 65°C aqueous, alkaline, acid, methanol, pepsin and trypsin extracts of strains O8 and O9 showed neither haemagglutination, nor haemagglutination inhibition; LPS, phenol phase of phenol-water extract and phenol-chloroform-petroleum ether extracts of these strains reacted only after heating; these findings confirmed the R-like character of antigens O8 and O9. Polysaccharides obtained by acetic acid treatment failed to give haemagglutination reaction; their haemagglutination inhibiting capacity was variable.

RESISTANCE FACTOR, PHAGE PATTERN AND ANTIGENIC STRUCTURE
OF *ESCHERICHIA COLI* ISOLATED FROM WATER AND FOOD

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The incidence in water and food of *Escherichia coli* strains playing an important part in the spread of R plasmids was examined. Serological and phage sensitivity tests showed that 43.4% of the strains belonged to "human enteropathogenic", 7.2% to "animal enteropathogenic", 10.4% to "human + animal enteropathogenic" serogroups. Antibiotic resistant strains occurred in 28.1%, multiple resistant strains in 2.7%; R-factor was present in 40.7% of the resistant strains. Resistance was especially frequent to ampicillin (60.8%), tetracyclin (41.9%) and chloramphenicol (29.7%). The commonest serogroups were O4, O18 and O148. Phage patterns of the strains usually corresponded to those of *E. coli* strains isolated from enteritis. In phage restriction there were some differences between the ϕ ⁻ R plasmids studied and R plasmids of enteritis strains.

BIOTYPES OF *BRUCELLA* IN HUNGARY

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Brucella strains isolated from 20 specimens of cow's colostrum, milk and foetus and from 8 specimens of pig foetus were determined using the methods of ALTON and JONES (1967) and of BRINLEY-MORGAN and GOWER (1966). Sixteen *B. abortus* strains belonged to biotype 1 and corresponded in properties to the Weybridge reference strain 544; biotype 2 was represented by 3, biotype 7 by 1 isolate. *B. suis* strains belonged to biotype 2.

SULPHUR HYDROGEN PRODUCTION BY *BRUCELLA*

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An attempt was made at explaining the contradictory data for H₂S production by *Brucella*. By the use of HUDDLESON's lead acetate paper strip method and of THIRY's MI-DI test, 18 *B. ovis* (17 routine isolates and *B. ovis*

63/290 reference strain) and *B. melitensis* "M 16" reference strain were examined. With MI-DI test all strains were shown to produce small, equal amounts of H_2S . The MI-DI test, according to experiments with aqueous H_2S solution, was about twenty times more sensitive than HUDDLESON's method. The value of species and type differentiation by H_2S production seems doubtful.

ISOLATION OF HAEMOPHILUS INFLUENZAE-MURIUM FROM THE RESPIRATORY TRACT OF MICE

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Type strains of *Haemophilus influenzae-murium* described by KAIRIES *et al.* (1936) and by IVÁNOVICS *et al.* (1937) were lost during World War II and the taxonomic position of the organism has since been doubtful. In 1973, a total of 23 strains were isolated from the nose and throat of CFLP white mice. Characteristic features of the isolates were as follows. Gram negative capsulated, non-motile, non-spore-forming, small coccobacilli or pleomorphic rods. X-factor required, non-haemolytic; capsule formation enhanced by DPN or ox serum; no growth on Endo or Löffler media, small colonies on egg medium. Aerobic, slight growth in CO_2 , optimum temperature $37^\circ C$. Nitrates reduced to nitrites; indole, H_2S , urease, gelatinase, oxidase, M.R., V.P. negative; catalase positive. Acid production without gas in glucose, laevulose, maltose, mannose, sucrose and trehalose but not in arabinose, dulcitol, galactose, inositol, lactose, mannitol, raffinose, rhamnose, sorbitol and xylose. The isolates were related serologically. Seronegative mice not harbouring the organism, after intranasal infection, developed fever and conjunctivitis then recovered; part of the animals became seropositive and carried the agent.

EFFECTIVENESS OF WAUTERS'S ENRICHMENT MEDIUM FOR THE CULTIVATION OF YERSINIA

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The growth of two strains representing *Yersinia enterocolitica* serogroups O3 and O9 was compared on bismuth sulphite, brilliant green, desoxycholate citrate (DC), eosin methylene blue and blood agar inoculated with 10^1 – 10^6 yersinia cells in faeces suspension. The best result was obtained with DC agar which allowed a recovery of 10^2 cells. Incubation at room temperature

for 48 hr was more favourable than at 37°C for 24 hr + at room temperature for 24 hr. WAUTERS's enrichment medium inoculated with pure cultures or with faecal suspensions and incubated at room temperature for 4 days, was inhibitory for the growth of yersiniae. In routine examination of faeces, WAUTERS's medium was advantageous: 10 out of 304 faecal specimens were positive with WAUTERS's + DC media, while direct inoculation of DC plates gave negative results.

A NEW MEDIUM FOR CULTURING ANAEROBIC BACTERIA

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A new medium suitable for the culturing of anaerobic bacteria from clinical specimens, pharmaceutical products and foods containing antibiotics and other antimicrobial agents has been elaborated. The meat infusion-yeast extract-glucose-starch medium is effective even after prolonged storage and is, therefore, applicable also as a transport medium. With slight modification it can be used for the determination of carbohydrate fermentation reactions.

SEROLOGICAL SURVEY OF MYCOPLASMA PNEUMONIAE INFECTIONS AND ANTIMYCOPLASMAL ACTIVITY OF ANTITUBERCULOTICS

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By growth inhibition test, serum antibodies to *Mycoplasma pneumoniae* were shown in 30.9% of 68 patients. Ten out of 16 patients with acute bronchitis and pneumonia, 2 out of 6 patients suffering from tuberculosis, but only 1 out of 12 patients with other disease were positive for *M. pneumoniae* antibodies. Antituberculotics (isoniazid, cycloserin, rigenicid, ethambutol, rifampicin) at therapeutic concentrations failed to inhibit the growth of human mycoplasmas. Erythromycin (0.02 µg/ml) was highly effective against glucose-splitting but not against arginine-decomposing mycoplasmas.

MYCOPLASMA PNEUMONIAE IN GYNAECOLOGIC DISEASES

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Eighteen patients with puerperal fever, adnexitis, vulvo-vaginitis, abscess of Bartholin's gland, erythroplasia and laceration of the portio were examined. *Mycoplasma pneumoniae* was isolated from 2, *M. hominis* from 3, *M. pneumoniae* + *M. hominis* from 2 patients. Growth inhibition test with the patients' serum showed the presence of *M. pneumoniae* antibodies in 12, *M. hominis* antibodies in 3, and *M. orale* 1 antibodies in 1 patient. All positive patients had a history of mild respiratory disease. The role of mycoplasmas in inflammatory gynaecologic diseases has been discussed.

MYCOPLASMA PNEUMONIAE IN DISEASES OF THE SKIN AND MUCOSA

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Mycoplasma pneumoniae antibodies were shown in the serum of 4 out of 7 patients with exudative erythema multiforme, 2 out of 4 patients with erythema nodosum, and of 1 patient with Mucha's disease. The latter patient and one with erythema nodosum had also *M. orale* 1 antibodies. A family infection due to *M. pneumoniae* was observed: the agent was isolated from the sputum of the patient and of his wife; *M. pneumoniae* antibodies were detected in their sera and in the serum of one child of the family.

MI-DI TEST FOR THE IDENTIFICATION OF ANAEROBIC BACTERIA

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THIRY's microdiagnostic (MI-DI) test based on biochemical reactions of logarithmic phase microcultures was compared with the classical H₂S, indole urease, gelatinase, glucose, lactose, sucrose, maltose, mannitol, and nitrate reactions. Clostridial strains maintained in collection and isolated from specimens were used. The MI-DI test has been employed with success also for identification of anaerobic spore-formers, with the following modifications to

ensure anaerobic conditions: (i) From plates incubated in anaerobic jar the colonies are washed off with pre-reduced physiological peptone water enriched with cysteine-HCl and micro-cultures are prepared in an anaerobic glove box. (ii) If no anaerobic facilities are available, micro-cultures can be prepared from 10–12 hr chopped meat broth cultures, but in this case the pH has to be readjusted to 7.0–7.2, since the acid production from glycogen interferes with the colour reaction. (iii) Micro-cultures are preferably incubated in anaerobic jars, but the pyrogallol method is also helpful.

UROGENITAL INFECTION IN CHILDREN

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Urine samples from 1664 children (862 boys and 802 girls) aged 6–14 years were cultured for aerobic and anaerobic bacteria and for *Candida albicans*. Parallel samples from 1055 children (598 boys and 457 girls) were seeded on Cled and McConkey slides of the Incubator set. At screening no special measures were taken; at repeated examinations middle-stream samples were collected after washing the external genitalia. At screening the specimens were sterile in 271 boys and 131 girls, positive in 833 children. Girls usually excreted a higher number of bacteria and the frequency of positivity increased with age. High bacterial contents were characterized by the predomination of *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus*; these organisms were frequently excreted together with *C. albicans*. Anaerobid bacteria were also often encountered. Of children showing significant bacteriuria at screening, 54% (mostly girls) excreted 10^5 or more organisms at the control examination. Results obtained with Incubator slides agreed with the usual methods in 85%.

ASSOCIATION OF PASTEURELLA MULTOCIDA AND RESPIRATORY DISEASES

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In two years *Pasteurella multocida* was isolated from 27 patients with non-tuberculous respiratory disease. Clinical data indicated that the organism may be regarded as an aetiological agent in such conditions.

INCIDENCE OF GRAM-POSITIVE ANAEROBIC COCCI
IN CLINICAL SPECIMENS

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Based on the classification of HOLDEMAN and MOORE (1972), 65% of 560 Gram-positive anaerobic cocci isolated from 1900 clinical specimens were identified. By morphological and biochemical characteristics, the strains were classified into 14 groups presumably corresponding to distinct species. Most strains were encountered in gynaecologic specimens and in deep inflammations. Peptostreptococci in pure culture were isolated from 18, members of the genus *Peptococcus*, from 8 patients. Anaerobic pathogens were detected in about three times more female than male patients, suggesting that the vaginal route played a primary role in the pathogenesis of anaerobic infections. Blood agar containing nalidixic acid (30 µg/ml) was shown to be the selective medium of choice for the isolation of Gram-positive anaerobic cocci. Peptostreptococci were sensitive to penicillin in 75%, to erythromycin in 95%, and to chloramphenicol in 93%. Peptococci were sensitive to these antibiotics in the range of 84–86%. All "Gaffky" isolates were penicillin sensitive. Tetracyclines exerted a moderate effect on the organisms tested.

PROPERTIES OF ESCHERICHIA COLI O114 ASSOCIATED
WITH A HOSPITAL OUTBREAK

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In the course of a hospital outbreak of enteritis affecting 112 infants and including 15 fatal cases, *Escherichia coli* O4 and O114 strains were isolated. The former produced no enterotoxin and varied in phage pattern. *E. coli* O114 strains showed positive ligated rabbit gut test, gave rise to specific IgM-type antibodies in the patients, and were identical in phage pattern and antibiotic sensitivity. Neither O4 nor O114 cultures caused keratoconjunctivitis in the guinea pig eye. *E. coli* O114 strains assumed to be responsible for the outbreak were identical in antigenic structure with type strain W26 O114:K90(B) and belonged to Ørskov's immunoelectrophoretic group 1Bb.

SERIAL BACTERIOLOGICAL EXAMINATION OF BRONCHIAL SPECIMENS

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A total of 50 patients was sampled by bronchial suction several times during hospitalization. The results were evaluated in view of reproducibility, sources of error and changes in bacteria colonizing the bronchi. The procedure of sampling caused no iatrogenic infections.

ANTIGENS OF STAPHYLOCOCCI SENSITIVE AND RESISTANT TO ANTIBIOTICS

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Staphylococcus aureus strains 211 and 223 isolated from hospital outbreaks and strain Wood 46 were trained to resistance to streptomycin, chloramphenicol, kanamycin and oxytetracycline. The resistant variants showed a significant decrease in the number of antigen-antibody systems and an increase in the amount of antigens with alpha-2 and beta mobility. With thin layer chromatography the resistant variants were shown to contain, in addition to monoglycerides, phospholipids (cephalin, isolecithin). The sensitive and resistant variants were compared for morphological characteristics and for haemolysin, pigment, coagulase, lecithinase and protease production.

COMPARISON OF MEDIA FOR THE CULTIVATION OF NEISSERIA GONORRHOEA

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Cervical, urethral and rectal specimens from 50 acute gonorrhoeal males and from 25 females with chronic (asymptomatic) gonorrhoea were seeded onto three kinds of prewarmed medium. After incubation at 37°C for 48 hr, specimens from males were uniformly positive on all media; of the specimens from females, 23 were positive on THAYER-MARTIN's medium, 20 on STUART's medium and 22 on AMIES's medium. Antibiotic-containing media were especially advantageous in chronic and rectal infections. Parallel inoculation of different media is recommended.

ROLE OF TRICHOMONAS VAGINALIS IN THE FAILURE
OF PENICILLIN THERAPY IN GONORRHOEA

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Failure of penicillin therapy in gonorrhoea is rarely due to the penicillin resistance of *Neisseria gonorrhoeae*. Non-gonorrhoeal female and male patients carried *Trichomonas vaginalis* in 22.5 and 3.0%, while gonorrhoeal females and males in 12.4 and 1.3%, respectively. Good therapeutic results were obtained if *T. vaginalis* positive gonorrhoeal patients were treated first with metronidazole then with penicillin. Microscopic observations *in vivo* indicated that *T. vaginalis* had incorporated gonococci and thus protected them against the effect of penicillin. Attempts at proving this finding *in vitro* have failed, since the two agents could not be grown on the same medium.

STAPHYLOCOCCAL INTERFERENCE IN PRACTICE

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Bacterial interference of apathogenic *Staphylococcus aureus* strain 502/A of SHINEFELD *et al.* with pathogenic staphylococci was examined. The patients received antibiotic therapy aimed against the infecting *S. aureus*, then nasal drops of 0.25 ml (5×10^5 cells/ml) living suspension of strain 502/A were given on five consecutive days. The patients became symptomless; relapses were observed only if the apathogenic organism had disappeared from their nose (*e.g.* after antibiotic treatment). Freedom of symptoms has been maintained in 37 patients for 4 years; administration of the apathogenic strain was safe.

Genetics and general microbiology

MAPPING OF δ -AMINOLAEVULONIC ACID DEHYDRASE AND
PORPHOBILINOGEN DEAMINASE LOCI

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Two genes (*hem B* for δ -aminolaevulinic acid dehydrase and *hem C* for porphobilinogen deaminase) involved in porphyrin synthesis by *Bacillus subtilis* were mapped. Transductants belonging to different recombinant groups

were separated partly by routine replica technique, partly by examining syntropism. The *hemA*, *hemB* and *hemC* loci were closely linked and situated between the *leuI* and *pheA1* loci. The *hem* genes were arranged in an order corresponding to the biosynthetic pathway.

ANALYSIS OF 16-3 PHAGE REGULATOR GENE IN RHIZOBIUM MELILOTI

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The 16-3 phage regulator gene of *Rhizobium meliloti* was analyzed by examining Cti4-Supr double mutants of the phage. Owing to the heat sensitivity of the repressor protein, the plaques of Cti4 phage became clear at 32°C. Twelve different Cti4-Supr double mutants were isolated, which were super-repressive as compared to Cti4: they produced turbid plaques at 32°C and clear plaques at 36°C. The nature of superrepressivity was examined with the following methods: (i) Immunity of lysogenic bacteria against homo-immune superinfection. (ii) The time needed for heat induction of the lysogen. (iii) Genetic mapping by two and three point crosses. The results indicated that (i) mutations 63A and 72A occurred at the promoter site as they fell on the edge of the regulator gene map and Cti4-63A and Cti4-72A double mutants exhibited a protracted heat induction time and high immunity of the lysogen against superinfection; (ii) the 27A mutation affected a point important for the binding to phage DNA of the phage repressor protein, since the mutant needed short heat induction time but caused an extremely high immunity of lysogenic cells; (iii) mutations 511A, 522A and 21A rendered the repressor more resistant to the inactivating effect of increased temperature.

IDENTIFIED GENES FOR PORPHYRIN SYNTHESIS IN BACILLUS SUBTILIS

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Streptomycin selection yielded numerous porphyrin auxotrophs from *Bacillus subtilis* 168 trpC. Part of them grew with δ -aminolaevulinic acid (ALA), the majority required haemin. By syntropism test the latter were classified into two different phenotypic groups: feeders accumulating ALA, and non-feeders accumulating porphyrins other than ALA. By transduction, the feeders were divided into two, the non-feeders into four subgroups. For each of the different *hem* loci, with one exception, a different enzyme involved

in porphyrin biosynthesis could be identified: *hemB* → ALA dehydrase; *hemC* → PBG deaminase; *hemE* → uroporphyrinogen decarboxylase; *hemF* → coproporphyrinogen oxidase; *hemG* → protoporphyrin iron chelatase.

PHAGE RESISTANT R-FACTOR MUTANTS WITH GENETIC TRANSFER ABILITY

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Mutants of *Escherichia coli* R 192.7 resistant to phage MS2 but retaining their genetic transfer ability were isolated at a frequency of 10^{-7} using nitrosoguanidine and selective isolation procedures. Derivatives carrying the mutant R-factor were characterized by (a) "fragility" of the pilus; (b) kinetics of MS2 phage adsorption and e.o.p. of phages MS2 and M13; (c) electron micrographs. One of the phenotypic group of mutants was unable to adsorb phage MS2; the 3 other phenotypic groups were characterized by denaturation, altered binding to the membrane, "crystallization" and non-random distribution of pili. Density of the original R 192.7 was much lower than that of F pili; it exhibited a characteristic UV adsorption spectrum. The pili consisted of 4 proteins (mol wt 15 000, 24 000, 26 000 and 39 000).

MECHANISM OF SPECIALIZED TRANSDUCTION BY RHIZOBIUM PHAGE 16-3

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Phage 16-3 specifically transduces a gene involved in cysteine biosynthesis in *Rhizobium meliloti*. The resulting *cys*⁺ transductants can be classified as (i) containing infective phages, segregating into *cys*⁺ and *cys*⁻ derivatives and yielding HFT lysate after induction; (ii) containing infective phages and failing to give HFT lysate; (iii) containing few phage genes, producing no *cys*⁻ segregants and failing to give HFT lysate even after superinfection with helper phages. The first class of transductants may be formed by site-specific recombination, the second by general recombinational mechanism, the third class by recombination eliminating a part of the phage genome essential for induction.

GENETIC AND BIOCHEMICAL STUDIES ON BACTERIOPHAGE V72
OF MYCOBACTERIUM SMEGMATIS VAR. RABINOWITZ

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Characteristics of phage V72 of *Mycobacterium smegmatis* var. *rabinowitz* examined in one-step growth experiments were: latent period, 65 min; average burst size, 60. Phage V72 closely related to *M. phlei* phage could be separated by polyacrylamide gel electrophoresis. The DNA of phage V72 obtained by phenol or sodium dodecyl sulphate treatment showed a linear structure in agarose gel electrophoresis. Mutants sensitive to 44°C were isolated by nitrosoguanidine and sodium nitrite treatment. The frequency of revertants was 5×10^{-4} – 5×10^{-5} .

EFFECT OF COLICINOGENIC FACTORS ON THE VIRULENCE
OF SHIGELLA FLEXNERI 2a

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Escherichia coli *colE1-ML* and *colV-K94* factors were transferred to a virulent *Shigella flexneri* 2a strain at 1–2% frequency. *S. flexneri* *colV*⁺ and *colE1*⁺*V*⁺ strains segregated *col*[–] derivatives: the *colE1* factor was incorporated stably. *S. flexneri* 2a *colV*⁺ lost its virulence to the guinea pig eye.

PLASMID EXPERIMENTS WITH SHIGELLA SONNEI

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Among 2500 *Shigella sonnei* isolates the incidence of antibiotic resistant strains was 61.6% in 1972–1973. Of the 75 resistant strains examined for the presence of R-factor, 99.5% carried *fi*[–] and 0.5% *fi*⁺ R-factors. Phage restriction was caused by 14 *col-R* strains and every plasmid complex carried I type R-factor. The R-factor *per se* caused no phage restriction; for this the plasmid-complex was probably responsible. As *col* and R-factors appearing together were incorporated into the recipient cells in 90.3%, it may be assumed that the two plasmids were linked.

SYNTHESIS OF VITAMIN B₁₂ BY VARIOUS SPECIES OF MYCOBACTERIA

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To determine the synthesis of vitamin B₁₂ by 5 species of *Mycobacterium* (*M. bovis* strain BCG, P. 1102; *M. phlei* ATCC 19249, *M. smegmatis* ATCC 14468, *M. fortuitum* ATCC 6841, *M. asiaticum* ATCC 25276), the correlation between phase of growth and amounts of vitamin B₁₂, and the influence of cobalt upon the synthesis, the strains were grown on Sauton medium containing CoCl₂ (100 µg/ml) and KCN. Assays were performed with cell-free extracts and culture filtrates using *Lactobacillus leichmannii* ATCC 7830 (tube method). All species produced vitamin B₁₂ both in bacterial extracts and in culture filtrates. Studies of correlation between growth phase and yields during long cultivation showed an optimum accumulation of vitamin B₁₂ in BCG and *M. phlei* at the 30th day. Cobalt did not influence the growth of BCG and *M. phlei*. The total yields of vitamin B₁₂ and production in cell-free extracts of BCG and *M. phlei* were markedly higher in the presence of cobalt (intracellular amounts of vitamin B₁₂ 101 µg/g dry wt cells and 76.2 µg/g dry wt, respectively). Vitamin B₁₂ levels for *M. smegmatis*, *M. fortuitum* and *M. asiaticum* were lower and cobalt failed to influence the results. The presence of methionine in samples was excluded by the use of the cup-plate method with *Escherichia coli* mutant 113-3.

PURIFICATION AND PHYSICOCHEMICAL CHARACTERIZATION
OF MYCOBACTERIOPHAGES

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Large amounts of *Mycobacterium butyricum* phage (A) and *Mycobacterium phlei* phage (B) were obtained in Sauton synthetic medium. The titre of the phages depended on the CaCl₂ concentration, the optimum was 0.001 M for A, and 0.004 M for B. First purification and concentration of the phage; were performed on a strong exchanger triethylaminoethyl-cellulose columns further concentration and purification, by stepwise CsCl gradient centrifugation following ultracentrifugation at 35 000 g for 60 min. Electronmicroscopically, the phage heads of A and B were identical in size (65 nm); while the tails were different in length (225 and 152 nm, respectively). Sedimentation constants were 490 s for A and 410 s for B. The molecular weights were calculated on the basis of electronmicroscopic dimensions (112 millions for A and 110

millions for B). The biological activity of the phages was preserved in the presence of Ca^{++} ions, while in sodium saline citrate (0.15 M NaCl and 0.015 M Na-citrate) it was lost irreversibly. Inactivation was also reflected by heat inactivation spectra and electronmicroscopic pictures.

REGENERATION OF BACILLUS MEGATERIUM PROTOPLASTS INTO BACILLARY FORM

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Lysozyme-induced *Bacillus megaterium* protoplasts were reverted into bacilli.

SERINE STARVATION IN RELAXED ESCHERICHIA COLI

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Upon serine starvation, as compared to histidine or arginine starvation, the relaxed mutant exhibits a lower RNA synthesis even in the presence of purine-pyrimidine bases and all other amino acids. When amino acid starvation was combined with folate cycle inhibition (trimethoprim and hydroxylamine treatment), there was no difference between the three amino acids in the amount of RNA synthesized. During amino acid starvation no guanosine tetraphosphate and MSII accumulation was demonstrated. The changes observed in ATP and GTP levels were not sufficient to explain the differences in the amount of RNA synthesized. The experiments yielded no explanation why a functioning folate cycle was necessary for RNA synthesis under amino acid starvation.

MS NUCLEOTIDE METABOLISM IN ESCHERICHIA COLI UNDER SERINE STARVATION

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Escherichia coli CP99 (serine, histidine and arginine dependent multi-auxotrophic stringent strain) showed a high guanosine tetraphosphate (ppGpp) and low guanosine pentaphosphate (MSII) level upon serine starvation, a low

ppGpp and high MSII level upon histidine starvation and a high ppGpp and medium MSII level upon arginine starvation. Serine-histidine double starvation resulted in a nucleotide level identical with that obtained upon serine starvation; addition of serine caused a rapid decrease in both ppGpp and MSII level and an increase in GTP level.

REGULATION OF NITRATE ASSIMILATION IN RHIZOBIUM MELILOTI

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In *Rhizobium meliloti*, a genetic correlation has been shown between the nitrogenase and inducible nitrate reductase enzymes. The latter was induced by nitrate or protein hydrolysate medium. Regulation of nitrate reductase was studied at different nitrate concentrations and redox conditions. Ammonium and chlorate exerted an inhibitory effect on this induction.

ASSOCIATION BETWEEN ADENINE NUCLEOTIDE SYNTHESIS AND VIRULENCE IN BACILLUS ANTHRACIS

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The adenylosuccinate lyase deficient mutant of *Bacillus anthracis* Vol-lum strain was avirulent to the mouse, although it remained capsulogenic and toxigenic. In the adenine-requiring mutant, nucleotide synthesis is ensured by a salvage pathway. The sonicated suspension synthesized adenine nucleotides from labelled adenine in the presence of exogeneous ATP. Ribose-5-phosphate was found to potentiate the nucleotide synthesis-stimulating effect of ATP. In the presence of exogeneous phosphoribosyl pyrophosphate (PRPP) the majority of labelled adenine was incorporated into adenine nucleotides. The results indicated that growth (adenine uptake) was probably limited by the exhaustion of the intracellular PRPP pool. Addition of PRPP failed to increase the growth rate significantly. Experiments *in vitro* and *in vivo* indicated that avirulence was due to a decreased growth rate of the mutants. The effect of defence mechanisms and of catabolic enzymes in the host could be excluded on the basis of results *in vitro*. Avirulence of the mutants was demonstrated also in mice treated with hydrocortisone. The decreased growth rate was due to a narrowing of the adenine nucleotide pathway and to certain catabolic enzymes such as alkaline phosphatase and adenosine deaminase

that decompose the synthesized adenine to substances not utilizable by the mutant.

STREPTOMYCIN DEPENDENT *ESCHERICHIA COLI*

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During streptomycin starvation, streptomycin dependent *Escherichia coli* forms non-septated filaments in complete medium. At 30 min after the addition of streptomycin the filament begins to fragment into rods. This fragmentation does not take place when protein synthesis is inhibited. Under streptomycin starvation, the streptomycin dependent strain might be lacking in some protein-like factor(s) necessary for cell division, production of which is inhibited at the translational level. In the presence of streptomycin, the ribosome is capable of normal translation.

APPLICATION OF PROTOPLASTS IN FUNGAL GENETICS

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(i) Enrichment of auxotrophic and temperature-sensitive mutants by selective protoplast formation is based on the observation that young cells are much more apt to yield protoplasts than old ones. Under certain conditions only the parental cells, and not the desired mutants, are able to grow: the former become physiologically "young" and, therefore, sensitive to enzymes degrading the wall, while the mutants remain resistant. If enzyme treatment is carried out in an osmotically stabilized medium, the sensitive cells give rise to protoplasts that are lysed on diluting the medium. Alternatively, this selective killing may be achieved directly by ensuring an osmotically unstable environment. In this case, it is possible to isolate the required mutants on selective media. The procedure is highly efficient and has wide applicability. (ii) Heterokaryon formation and nutritional complementation of auxotrophic mutants can be achieved with *Geotrichum candidum* through protoplast fusion and regeneration of the fused protoplasts. Complementation occurs at a frequency of 0.6×10^{-6} to 1.6×10^{-6} per protoplast pair. Under normal conditions, no anastomosis and heterokaryosis or sexual processes are detected in these mutants. (iii) A significantly increased fusion frequency of protoplasts of *Aspergillus nidulans* auxotrophic mutants could be attained under conditions favourable for the aggregation of protoplasts. The average frequency

of complementation was 2.5×10^{-3} as calculated on the basis of complementation per colony forming unit. The results indicate further possibilities concerning the intraspecific and interspecific transfer of the nuclear and mitochondrial genetic material in fungi.

EFFECTS OF METHICILLIN ON LIPID AND CELL WALL SYNTHESIS IN METHICILLIN-SENSITIVE STAPHYLOCOCCUS AUREUS

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The effect of methicillin at 1 $\mu\text{g/ml}$ ($1/2$ MIC) and 2 $\mu\text{g/ml}$ (1 MIC) concentration on growth dynamics, ultrastructure, lipid and nucleotide-bound peptidoglycan precursor contents of *Staphylococcus aureus* 5814S growing exponentially have been studied at 0, 1 and 2 hr intervals. The addition of $1/2$ MIC methicillin to the cultures, although it allowed a significant gain in cell number as related to the 0-time value, resulted in a significant decrease in viable count as compared to the control. The structure of cross-walls was considerably different from that of untreated cells. The amounts of lipids of all classes were significantly elevated. Amino-phospholipids involved in cell wall synthesis and nucleotide-bound peptidoglycan precursors showed a significant accumulation. After adding 1 MIC methicillin, the number of viable cells remained at the initial level. Organization and control of cross-wall formation and cell division seemed to suffer a serious damage. No considerable decrease in total lipid content occurred; the amount of polar lipids including lysyl-phosphatidylglycerol, dropped significantly after 2 hr exposure to methicillin. In accordance with the ultrastructural changes, at 1 MIC more cells wall precursors accumulated than at $1/2$ MIC.

EFFECT OF RIFAMPICIN ON THE R-FACTOR IN ESCHERICHIA COLI

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Rifampicin at 0.19–3.0 $\mu\text{g/ml}$ concentration inhibited R-factor transfer from *Escherichia coli* K₁₂J5 RC 709 F[−] prol[−] meth[−] Sm^r T^r Su^r Cl^r to *E. coli* K₁₂ Sm^s T^s Su^s Cl^s. The recombination frequency decreased parallel with the increase of rifampicin concentration; above 3 $\mu\text{g/ml}$ the growth of both the donor and the recipient strain was inhibited. Rifo and rifamycin acted similarly as rifampicin: they inhibited the transfer of R-factor, but failed to elimin-

ate it. The multiple resistant recombinants were used as donors in conjugation experiments with *E. coli* K₁₂ W-1 A₂ L⁻ Na-azide^r recipient. At a concentration of 4-6 µg/ml, rifampicin failed to delete the R-factor from the multiple resistant recombinants.

SEPTA OF DERMATOPHYTE MACROCONIDIA

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Although macroconidia play a basic role in dermatophyte taxonomy, their important parts, the septa, have been neglected by both morphologists and physiologists, as well as by taxonomists. Under the light microscope there was no difference in the septa between *Microsporium cookei*, *M. felineum*, *M. gypseum*, *M. nanum*, *M. racemosum*, *M. umbonatum*, *M. vanbreuseghemii*, *Epidermophyton ajelloi* and *E. floccosum*. In these species the septum seemed to consist of three layers, of which only the middle one can be regarded as a true septum. The two others correspond to the inner layer of the macroconidium wall initially covering the macroconidial plasma, invaginated by the septum growing inwards from the middle layer of the macroconidial wall, or from the inside surface of this layer. These two septal layers are thus parts of a chamber wall. This structure differs from the septa of filaments, the only similar structure discussed in the literature. Optical phenomena disturbing light microscopic observations have been demonstrated on glass-models of macroconidia.

Agricultural and industrial microbiology

STRAIN VARIATIONS IN RHIZOBIUM JAPONICUM; THE EFFECT OF INOCULATION WITH MIXED CULTURES ON SOYBEAN YIELDS

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Forty-seven strains of *Rhizobium japonicum* were isolated from root-nodules of soybean plants. They were classified according to their rates of nodule formation and nitrogen fixation into 4 groups: parasitic, slightly effective, effective and highly effective. The effect of the isolates, as well as that of 2 other local and 3 foreign strains, varied according to the kind of soybean cultivar used (Clark, Hampton, Jackson and Rebel). Growth of *R.*

japonicum was inhibited during the germination of soybean grains, but in a yeast extract deficient medium their growth was highly stimulated by the development of roots. In a field experiment, yield, nitrogen and oil content of Hampton soybean grains were significantly increased by inoculation with mixed strains of *R. japonicum*. Gains due to inoculation were improved by field applications of nitrogenous and phosphoric fertilizers.

ROLE OF MICROBIOLOGICAL OXIDATION OF IRON IN BIOMETALLURGY

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In acid medium, ferrous iron is oxidated to ferric iron slowly or not at all. The process is catalyzed by certain chemolithrophic bacteria of the genus *Thiobacillus*. Ferric iron obtained in this manner is an active extracting agent: when reduced to bivalent iron, it oxidizes metals in ores and makes them soluble as sulphates. In enriched, mixed cultures of predominantly *T. thiooxidans* and *T. ferrooxidans* originating from iron-containing acid waste water, optimum yields were obtained at 34–37°C under aeration; during incubation the pH gradually decreased, the Eh and the amount of ferric iron increased. Cultures active at pH 2.0 and at high iron concentrations (44 g Fe/litre) were produced in ferrosulphate medium. For the biometallurgical process, a solution containing 10–20 g ferric iron per litre (pH 2.0–2.5, Eh +600 mV) is suitable. After a 1–3 hr treatment of ores (from Gabon Africa) containing uranium and vanadium, 77–94% of the former and 25–40% of the latter leached out. The procedure is suitable also for other metals. The iron-containing extracting solution, after recovery of the soluble metal (U, V, etc.) can again be used after microbiological reoxidation.

MICROFLORA OF COLA-TYPE REFRESHMENT DRINKS

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Cola-type tonics contain special ingredients to prevent microbiological spoilage. Experiments were performed to estimate changes in the starting number of microorganisms during storage. Ten specimens were taken at intervals from each batch of various Cola-type tonics of various makes. (i) Mesophilic aerobic bacteria and coliforms occasionally present in the fresh product multiplied moderately. Viability of coliforms persisted for one month,

of the mesophilic aerobic bacteria, for one year. (ii) Yeasts grew rapidly and abundantly, but lost their viability in one year. (iii) Molds did not multiply. (iv) Pollution with lactobacilli was unfrequent, these organisms disappeared in a short time.

SEASONAL CHANGES IN EXTRACELLULAR-ENZYME-PRODUCING MICROFLORA IN A PHYTOCENOSIS

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Quantitative seasonal changes in microorganisms decomposing large-molecular organic substances were examined in two phytocenoses (*Festucaetum vaginatae danubiale* and *Convallario-Quercetum roboris danubiale*) of the Csévharaszt reservation and in an agricultural area (*Secaletum cultum*). The number of microorganisms producing extracellular enzymes decomposing cellulose, pectin, starch, chitin, gelatin, casein, urea and esculin was determined at monthly intervals. During a one-year cycle two maximum and two minimum values were observed: in oak-wood soil a higher peak (December-March) and a lower peak (June); in the grass soil two identical rises in the same seasons. As regards total microbial counts, the phytocenoses fell in the order: oak $\gg$ grass $>$ rye-field. Experiments performed on minimal medium revealed that the change in the number of decomposing microorganisms in rye-field took a reverse course as compared to that in oak-wood, attributable to an upsetting of the biological balance by agriculture.

HORMONAL CHANGES IN CORN TISSUE INFECTED WITH *USTILAGO MAYDIS*

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In the incubation period, during the generalization of *Ustilago maydis*, growth of the plant is inhibited. This is independent of hypertrophy, as it occurs also in plants infected with monosporidial isolates causing no tumour. Phenol-type toxic substances present in corn tissue act on the parasite and metabolite release; in consequence of a protein-turnover damage in the host, they give rise to partial protein hydrolysis. The free amino acid and amide level thus increases considerably; from tryptophan of the stored amino acid pool, indole acetic acid is formed. The increased auxin level inhibits the normal course of morphogenesis and promotes ethylene production in the

tissues, meanwhile the level of gibberellins decreases. Changes in ethylene production and gibberellin level lead to hypertrophic tissue transformations. The tumours increase in size after ether treatment and decrease after gibberellin treatment, indicating an antagonism between the two substances.

WHEAT DISEASE CAUSED BY FUSARIUM SPECIES IN HUNGARY

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In 1970–1973 different districts, situated mainly on the Hungarian Plain, were sampled for *Fusarium* species. The 658 isolates identified by BOOTH's system (1971) belonged to 14 different species. Ninety per cent of the isolates belonged to *F. culmorum* and *F. graminearum*; other species encountered were *F. avenaceum*, *F. flocciferum*, *F. oxysporum*, *F. moniliforme*, *F. poae*, *F. acuminatum*, *F. tricinctum*, *F. fusaroides*, *F. semitectum*, *F. solani*, *F. sporotrichoides* and *F. sambucinum*. *F. graminearum* caused changes mainly in the head region (seed, cob and glumellae), while *F. culmorum* occurred on all parts of the plant from the root to the head. All plants examined showed evidence of disease.

SELECTION OF X VIRUS-FREE POTATO GENERATIONS

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To detect potato leaf roll, a disease most dangerous to potato production in Hungary, the winter cuttings are examined. For Kiszárda pink potato, the December survey resulted in a 48%, the February one in a 68% selection of diseased roots. At field production, however, still 27% diseased plants were encountered. "Gül Baba" potatoes showed a lower degree of infection. The antiserum method and especially the *Gomphrena globosa* test-plant method gave satisfactory results: the former revealed 0.2%, the latter 1% incidence of X virus infection. At present X virus infection occurs in the stock material in less than 0.1%.

PATHOGENICITY OF FUSARIUM SPECIES ATTACKING CORN

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During 4 years, about 1000 corn fields situated in 200 different districts of Hungary were surveyed for fusarium infection and the diseased plants were sampled. The disease occurred all over the country; from corn-ears 19, from corn-stalks 17 *Fusarium* species and subspecies were isolated. In corn-ears the most frequent organisms were: *F. moniliforme* Sheld var. *subglutinans* (Wr. et Rg.) (28.1%), *F. graminearum* (Schwabe) (22.9%), *F. culmorum* (W. G. Sm.) Sacc. (9.5%), and *F. moniliforme* Sheld (8.1%). In stalks *F. moniliforme* Sheld var. *subglutinans* (Wr. et Rg.) (14.8%), *F. culmorum* (W. G. Sm.) Sacc. (12.3%) and *F. sambucinum* Fuck (11.6%) predominated. *F. gibbosum* App. et Wr. emend Bilai (9.4) and *F. sporotrichiella* Bilai (9.2%) were also frequently isolated. Comparative pathogenicity tests on corn-ears showed considerable differences between the isolates: of the 17 species (variants) tested, 5 were highly, 5 moderately, and 7 slightly pathogenic.

GROWTH KINETICS AND RELEVANT LIPID COMPOSITION
OF RHODOTORULA AND CANDIDA

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Data for growth of *Rhodotorula* and *Candida* were plotted on growth rate curve ($\frac{dx}{dt}$ as a function of x concentration) by the use of the mathematical model of KONO and ASAI. To obtain an ideal growth curve, the kinetic constants were checked by computer simulation. The model allowed an estimation of slight differences in growth parameters (aeration, stirring, changes in pH and temperature). Gas chromatographic studies indicated an association between saturated-unsaturated fatty acid content and carotin (torulin, torulin hydroxide, tolurarrhodine) production in *Rhodotorula*. Under optimal growth conditions, primarily C_{18} fatty acids with two or three unsaturated bonds (linoleic, linolenic acids) were produced, while the amount of saturated stearic acid decreased. In *Candida* the ratio of saturated-unsaturated C_{16} fatty acids (palmitic and palmitoleic acids) depended on the degree of aeration.

Immunology

STABILITY OF BORDETELLA PERTUSSIS VACCINES

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The decrease in the potency of pertussis vaccines was estimated by various authors at 1–40% yearly. The discrepant results may have been due to the periodicity of potency decrease ($S_f < S_y$). The standard deviation of active mouse protection test used for the assay, the virulence and the bacterial count of the challenge suspension were not responsible for the periodicity. Of measurable parameters of production and control (toxicity, sensitizing capacity to histamine), only the quality of casein hydrolysate, an ingredient of the culture medium, seemed to be associated with the periodic decrease in potency. Analysis of periodicity functions indicated that some unknown factors may also be involved.

STABILITY OF BORDETELLA PERTUSSIS SUSPENSIONS

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The mean potency of 53 acid precipitated polyvalent bulk suspensions of *Bordetella pertussis* produced during 13 years was, in at least three potency assays, 19.45 IU/30 IOU (1 sd $\pm$ 14.66 IU/30 IOU). The potency of the suspensions decreased in 5–9 years below the WHO and Hungarian minimum requirement 4 IU in a single human dose. The potency increased for one year after preparation, then decreased to half of the original value after an average of 8 years. The annual potency decrease plotted against time gives a logarithmic function. Using this function, the expiry date of new batches can be estimated.

EXAMINATION OF PERTUSSIS ANTIBODIES WITH COMPLEMENT FIXATION TEST

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Bordetella pertussis antigens for complement fixation (CF) test were made from 3 strains using four different preparations (whole cell suspension, saline, cryolytic and alkaline extracts) and compared with one another. The optimum density was 5 T/ml for bacterial suspensions; equivalent extracts

were prepared from 20–50 T/ml cultures. The different antigens gave results almost identical with those of 55 serum specimens from vaccinated children. For practical and economic reasons the whole cell suspension is the antigen of choice. There was no parallelism between CF and tube agglutination titre of the serum from vaccinated children and from patients with suspected pertussis. For the diagnosis of pertussis both reactions are recommended, since in about 15% of the patients only the CF test was positive.

VACCINATION AGAINST TRANSMISSIBLE GASTROENTERITIS (TGE)
OF SWINE. TITRE AND PERSISTENCE OF VIRUS NEUTRALIZING
ANTIBODIES IN THE SERUM AND WHEY OF VACCINATED SOWS

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Twenty-seven sows were vaccinated 9–27 days before farrowing. Serum and whey samples were taken after farrowing and examined by virus neutralization test. Antibody levels equivalent to those found in suckling piglets in TGE epidemics at the time when the epidemic had ceased, were reached 27 days after vaccination. In 9 sows examined at the end of lactation, on the day of farrowing the whey antibody titre was $1 \log_2$ higher than that of the serum. During the subsequent 3–5 days it decreased by 1 or $2 \log_2$ and remained at that level to the end of the 35-day long lactation period. In another experiment, 137 sows of a large farm were vaccinated once or twice in a two or six-week interval. Then during a six-month period, blood samples were collected at 2–4 weeks intervals and titrated for virus-neutralizing antibodies. The highest level and the longest persistence of antibodies was achieved by means of double-dose vaccination at an interval of two weeks; maximum values occurred 8 weeks after vaccination (average $6.87 \log_2$) and the titres remained practically constant for six months (average $6.50 \log_2$).

EFFECTIVENESS OF TRIVALENT FOOT-AND-MOUTH DISEASE VACCINE

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Four large herds of cattle immunized with different vaccines against foot-and-mouth disease were sampled for virus neutralizing antibodies. The degree of immunity was estimated by the S-index method. The first serum specimens were taken 6 months after the primary vaccination, the second

sampling was performed 3 weeks after the booster vaccination. Animals developing immunity to virus types O, A and C after the first dose were distributed as follows: WALDMANN vaccine 47, 40 and 87%; FRENKEL vaccine 53-60, 53-67 and 100%, respectively. After the second immunization, both vaccines ensured a 100% immunity. The highest degree of immunity was observed to type C after the first and also after the second dose. After booster vaccination there was no significant difference in S-index between the herds. It is concluded that FRENKEL's method of vaccine production is an adequate substitute of WALDMANN's method.

VIRUS CONTENT AND EFFECTIVENESS OF FOOT-AND-MOUTH DISEASE VACCINES

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After fluorocarbon precipitation of non-immunizing 7 nm virus components, the amount of 22 nm particles was determined by 50% haemolytic end-point quantitative complement fixation test. The effective virus concentration in the lingual epithelium of cattle, used for the production of C-type vaccine, varied from sample to sample. Effectiveness of the vaccines was examined in adult mice and was expressed as the ratio of titres for the adapted virus strain in non-vaccinated and vaccinated animals (E index). In commercial monovalent C vaccines the complement fixation activity of 22 nm particles and the immunizing potency showed a logarithmic regression: $E \text{ index} = -0.143 + 2.27 \log \alpha GF$.

LYMPHOCYTIC CHORIOMENINGITIS VIRUS INFECTION IN GERM-FREE MICE

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Intracerebral lymphocytic choriomeningitis virus infection showed a different course in CD-1 mice raised germ-free and in the conventional group. Six-week-old animals were injected with 100 LD₅₀ of WE virus strain. Animals in the conventional group exhibited characteristic symptoms and died in 7-9 days. In germ-free mice the symptoms appeared later and only 60% of them died in 7-12 days. From the brain and blood of the surviving mice the virus was recovered on the 21st day after infection. The results indicate a decreased cellular response in germ-free mice, probably associated with their underdeveloped lymphoid system.

ROLE OF COMPLEMENT IN BACTERIAL IMMUNITY

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Resistance and immunity to infection were examined in mice de complemented with aggregated human gamma globulin and C4 inactivator. Infection with *Staphylococcus aureus* Smith strain and with *Vibrio cholerae* Ogawa strain resulted in a high increase of resistance in mice treated with aggregated gamma globulin: members of this group survived while all control mice died. The striking result cannot be explained by a de complementation, since treatment with C4 inactivator caused a suppression of immunity irrespective whether the complement level was decreased at the afferent or at the efferent site of the immune reflex.

FACTORS INHIBITING ENTERIC ESCHERICHIA COLI INFECTION

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Factors protecting the rabbit against ingested *Escherichia coli* (free hydrochloric acid in gastric juice, rapid peristalsis in the small intestine inhibitory mechanisms in the caecum) were suspended by neutralization with sodium carbonate and by stopping peristalsis by ligation of the gut in part of the animals and with intravenous endotoxin injection in others. All kinds of treatment resulted in 24 hr in a considerable increase in the number of the rabbit's own *E. coli* bacteria, indicative of the role of the above mechanisms in the protection against pathogenic *E. coli*.

IMMUNOLOGICAL RESPONSE IN THE CHICKEN

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Although lymphocytes released from the thymus or bursa are regarded as immunologically mature, and "B" cells are present in the first 4-5 weeks of life, the chicks fail to produce antibodies to a number of antigens. Chicks were immunized twice with bovine serum albumin at the age of 2 weeks and 4 weeks later. The control group received one injection at the age of 6 weeks.

Bursa-dependent folliculi (BF), absent in non-immunized chicks, showed a definite proliferation 4 days after injection of the antigen; and no antibodies were demonstrated. Antibodies appearing after immunization were in 50–80% of the IgG-type, while in the control group mainly IgM and at most 5–20% IgG antibodies were present. The IgM antibody titre in the reimmunized group was lower than in the control. Immunization in “immature” age can, accordingly, induce IgG memory without a primary antibody response, and bring about a partial tolerance of IgM-producing cells. The ontogenesis of B cells seems to have a stage influenced by the antigen; this is probably associated with BF production. This compartmentalization plays some role not only in the development of immunological memory, but also in promoting the antibody-producing capacity of B cells.

EFFECT OF ENHANCING FACTOR ON ANTI-TETANUS IMMUNITY IN MICE

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Enhancing factor was produced from the spleen and thymus of mice immunized with two doses of tetanus toxoid adsorbed to aluminium hydroxide. The tissue culture medium was purified on Sephadex G-150 column. Each protein fraction was given to mice together with toxoid and the developing immunity was compared with that of the control group treated with toxoid alone. Fraction I obtained from the spleen of immunized animals was stimulatory; a single toxoid + fraction I injection was equivalent with the two toxoid infections. Fractions II and III exerted no stimulatory effect.

STUDIES ON ANTIPYROGENIC ACTIVITY

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Treatment with *Escherichia coli* O83 immune serum or active immunization with *E. coli* O83 vaccine decreased the pyrogenic effect of *E. coli* O83 Boivin extract. The effect was similar when *Salmonella enteritidis* vaccine, immune serum and Boivin extract were used. The sera and active immunization exerted no cross-protection against the two Boivin extracts. Antipyrogenic immune serum against *S. minnesota* Re LPS did not reduce the pyrogenic effect of heterologous *E. coli* O83 or *S. enteritidis* Boivin extract. In rab-

bits immunized actively with *S. minnesota* Re vaccine, the pyrogenic effect of *S. enteritidis* Boivin extract was reduced more markedly than that of *E. coli* O83 Boivin extract. Active immunization with *E. coli* O83 or *S. enteritidis* vaccine did not decrease the pyrogenic effect of *S. minnesota* Re LPS. In spite of the seemingly similar biological effect of endotoxin of Gram-negative bacteria when injected parenterally, it may be supposed that the antigenicity of the structure responsible for pyrogenicity varies with the individual bacteria.

ROLE OF BILE ACIDS IN THE PHYSICO-CHEMICAL DEFENCE MECHANISM OF THE MACROORGANISM

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Under normal conditions, bacterial endotoxins are not absorbed from the intestinal tract of rats. By continuous cannulation of the common duct, rats were deprived of bile. Endotoxin given orally to these animals produced a shock. The rats were protected against the shock by bile or bile salts. This effect of bile acids is associated with their detergent activity: bacterial lipopolysaccharides are deteriorated into non-toxic fragments. Bile acids act similarly on other lipid and lipoprotein substances, as *e.g.* on the coat (envelope or peplon) of sodium deoxycholate sensitive virus particles. The protective effect of bile acids has been termed physico-chemical defense mechanism.

EFFECT OF NDV ON PRIMARY AND SECONDARY IMMUNE RESPONSE OF HAMSTERS

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In the spleen of hamsters treated first with NDV then with sheep erythrocytes the number of cells producing 19S haemolytic antibodies decreased. As NDV is an effective interferon producer in the hamster, the immunosuppression was assumed to be associated with interferon production. When NDV was given 6 days prior to a second dose of erythrocytes, in course of the secondary immune response the number of cells producing 19S and 7S haemolytic antibodies was significantly lower than in the control animals. A decrease of the primary antibody response occurred during the 10 days following the erythrocyte injection in NDV infected hamsters. NDV seems to inhibit the secondary antibody response only for 2-3 days.

THE LYMPHOID SYSTEM OF MICE DYING OF ACUTE TOXOPLASMOSIS

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Forty 6-week-old CFLP mice of both sexes were injected intraperitoneally with 25 000 cells of *Toxoplasma gondii* strain RH. The lymphoid system was examined in 20 mice sacrificed on the 7th day, in 20 mice died in acute toxoplasmosis on the 7th–8th day, and in 20 control mice. In mice dying of acute toxoplasmosis there was a definite hypertrophy of the spleen (spleen index 1.81), a severe atrophy of the thymus (thymus index 0.23) and a strikingly low circulating lymphocyte count (86% decrease as compared to the control).

PERSISTENCE OF BCG STRAINS IN THE SPLEEN OF MICE

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In the BCG control programme of the WHO, two subsequent studies have been performed on the persistence for 1 year of 10 different BCG substrains. Persistence is associated with the residual virulence of the strains and offers adequate information as to the selection of the culture for vaccine production. On the basis of overall grouping of inoculated and recovered live bacterial counts, the substrains were classified into three groups. (A) Intensely persisting cultures, exp. 1: (1) Brazilian, (2) French, (3) Moscow; exp. 2: (1) Moscow, (2) French, (3) Swedish. (B) Intermediary cultures, exp. 1: (4) Swedish, (5) Danish, (6) Ciba, (7) Glaxo; exp. 2: (4) Brazilian, (5) Danish, (6) Glaxo, (7) Ciba. (C) Moderately persisting cultures, exp. 1: (8) Vole, (9) Prague, (10) international reference; exp. 2: (8) international reference, (9) Japanese.

LISTERIA INFECTION IN MICE

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Graded doses of virulent (i.p. $LD_{50} = 2.3 \times 10^7$ cells) and slightly virulent (i.p. $LD_{50} = 1.5 \times 10^9$ cells) *Listeria monocytogenes* cultures were given to mice intraperitoneally (10^3 – 5×10^5 cells) and orally (10^4 – 10^7 cells). Virulent

organisms persisted in the peritoneal cavity and organs longer than less virulent ones. Septic condition with positive heart blood culture was caused only by the virulent strain. After oral infection with 10^9 cells, virulent listeriae were excreted in the faeces longer than avirulent ones. Oral doses of 10 virulent cells caused illness with ruffled fur and a loss in weight; part of the animals died. After ingesting the same dose of the slightly virulent culture, there was only a loss in weight. The higher capacity of the virulent strain to grow in the tissues is similar as with salmonellae.

MOUSE PATHOGENICITY OF *LISTERIA MONOCYTOGENES*

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The invasive capacity of *Listeria monocytogenes* increases 80–500 times in mice treated parenterally with iron-dextran. In mice immunized with living listeriae, iron-dextran had no such effect. The mode of action of iron-dextran is similar as that of the heat-resistant sonicated listeric extract termed Mortality Enhancing Factor. In subsequent experiments mice immunized with listeriae were infected with *Erysipelothrix rhusiopathiae* and, *vice versa*, animals immunized against swine erysipelas were infected with *L. monocytogenes*. In the former group a fatal infection (somewhat slower in course than in non-immunized mice) developed, while in the latter group a considerable protection was observed.

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BUDAPEST, AUGUST 25-30, 1974*

**APPLICATION OF ENZYMES IN THE TRANSFORMATION OF ORGANIC
COMPOUNDS**

*** Organized for the Federation by the Hungarian Biochemical Society**

REVIEWS

THE SCOPE AND POTENTIAL OF ANTIBIOTIC CONVERSION BY MICROORGANISMS

OLDRICH K. SEBEK

The Upjohn Research Laboratories, Kalamazoo, Michigan, U.S.A.

Summary. Almost 50 antibiotics have been reported to be modified microbiologically and the changes observed were grouped into 16 types of reactions. Most of the products of the reactions were antibiotically inactive, several have assumed a considerable clinical significance and others are of real or potential economic value. The catalysis in most instances has been effected by whole cells and in a few cases the respective enzymes were isolated, purified and crystallized.

Aside from the age-old process of making vinegar from ethanol and microbial resolution of the racemic mixture of tartaric acid by Pasteur, the oxidation of glucose to gluconic acid [1], the dehydrogenation of sorbitol to sorbose [2], of mannitol to fructose [3], or glycerol to dihydroxyacetone [4], may be cited as the first examples of well-defined simple microbial conversions of organic substances.* Since then many other changes that are catalyzed by microorganisms, have been observed. Enzymatic reductions have been carried out [cf. 6, 7] and the asymmetric conversion of benzaldehyde to acetylphenylcarbinol by yeast [8] has become of practical importance in the synthesis of L-ephedrine. The value of microorganisms in chemical syntheses was dramatically enhanced when the microbial 11 α -hydroxylation of progesterone [9] resulted in an economic production of chemotherapeutically important steroid hormones. The success of this reaction triggered massive programmes in which many thousands of microorganisms have been tested for their usefulness in the modification of the steroid molecules. It also spurred similar investigations of other substances, and many reports have since been published on microbial conversions of straight-chain and cyclic hydrocarbons, aromatics, terpenes and alkaloids, as well as of pesticides and antibiotics [cf. 10]. The primary objective of this kind of work on pesticides has been their detoxification, and on others the discovery of novel and useful products. To accomplish the latter, the search for new antibiotics through systematic screening has been supplemented by their chemical modification. It is, however, noteworthy that chemical modification

* Today, bacterial dehydrogenation of D-sorbitol to L-sorbose is an important step in the synthesis of ascorbic acid which is produced in 11 million pound quantities and valued at \$ 18 million in the U.S. alone [5].

Table I

Microbial conversion of antibiotics

1. *Acylation*
Chloramphenicol [13, 14], neomycins, kanamycins, gentamicins, sisomycin [15], cycloheximide [16], spiramycin [17], T-2636 antibiotics [18].
2. *Phosphorylation*
Kanamycins, streptomycin, dihydrostreptomycin, bluensomycin, neomycins, paromamine, paromomycin, gentamicins, butirosin, ribostamycin [19], lincomycin [20], clindamycin [23], tubercidin [22].
3. *Adenylation*
Streptomycin, mannosidostreptomycin, bluensomycin, spectinomycin, gentamicin C₁ [19], clindamycin [23].
4. *Demethylation*
Griseofulvin [24], lincomycin, clindamycin [25].
5. *Dehydrogenation*
Dihydroabikoviromycin [26], griseophenone A [27].
6. *Carboxymethylation*
Ribostamycin [28].
7. *Hydroxymethylation*
N-Demethylclindamycin [29], griseofulvin [30].
8. *Oxidation, hydroxylation*
Fusidic acid [31], rifamycin B [32], griseofulvin [30, 33], formycin B [34], mycophenolic acid [35], daunomycin [36], maridomycin [37], 5a,11a-dehydrochlortetracycline [38].
9. *Sulfoxidation*
Lincomycin, clindamycin [25].
10. *Epoxidation*
cis-Propenylphosphonic acid [39].
11. *Hydration*
Toyocamycin [40], 5a,6-anhydrotetracycline [41].
12. *Reduction*
Chloramphenicol [42], tylosin [43], dehydrogriseofulvin [33], 5a,11a-dehydrochlortetracycline [38], ascochitine [44], maridomycin, josamycin [45], nisin, subtilin [80].
13. *Deamination*
Formycin A [46], puromycin aminonucleoside [47].
14. *Hydrolysis*

<i>Lactone</i>	Actinomycin, echinomycin, etamycin, staphylomycin S, stendomycin, vernamycin B ₂ [48], antimycin A [49], cordycepin [50];
<i>Lactam</i>	Penicillin, cephalosporin [51];
<i>Amide</i>	Penicillin, cephalosporin [52], novobiocin [53], bleomycin B ₂ [54], chloramphenicol [55], polymyxins, tyrocidine [56], gramicidin S [57];
<i>Ester</i>	Cephalosporin C [58], bundlin B [59], leucomycin [60], maridomycin [61].
<i>Glycoside</i>	Mannosidostreptomycin [62].
15. *Isomerization*
Showdomycin [63].
16. *Transglycosylation*
Neamine, kanamycin A [64], monensin [65].

of benzylpenicillin became eminently successful only when microbial methodology provided the crucial intermediate, 6-aminopenicillanic acid. In a similar way, analogues of aminoglycoside antibiotics have been prepared chemically, which are highly resistant to and therefore highly effective against, enteric pathogens. Their preparation was made possible only when microbiological and enzymatic studies had identified the manner in which these enteric bacteria inactivate the parent aminoglycosides (acetylation, phosphorylation, adenylation). In the wake of these successes, other antibiotics were investigated in this way and, to date, almost 50 of them have been reported to be modified microbiologically. They can be grouped into 16 types of reactions and are listed in Table I. Additional discussion of these reactions may be found in our earlier reports [11, 12].

As interesting as these data may be, they are disappointing from the practical point of view. Almost all of the examples listed in Table I yielded antibiotically inactive compounds. Only a few gave desirable products: N-demethylated clindamycin [25]; 5-hydroxy-7-chlortetracycline; adriamycin, a valuable antineoplastic agent, which is a 14-hydroxylated daunomycin and produced by a mutant of the daunomycin-synthesizing streptomycete [36]; and the above examples (6-aminopenicillanic acid and esters of the aminoglycoside antibiotics) which in turn led to the chemical preparation of valuable semisynthetic analogues and derivatives of the parent compounds.

Most of the conversions listed in Table I were carried out by whole cells and their enzymatic aspects were studied in those instances in which practicality has been demonstrated: β -lactamases (penicillinases) hydrolyze the β -lactam portion of penicillin to penicilloic acid. These enzymes have been investigated in detail because they are produced by penicillin-resistant staphylococci and Gram-negative bacteria, and are therefore of considerable clinical significance [66]. The microbial inactivation of chloramphenicol, aminoglycoside antibiotics and of spectinomycin has been studied extensively for the same reason [67]. Their inactivation is due to the ability of certain enteric pathogens such as *Salmonella* and *Shigella* and of *Escherichia coli* to acetylate, phosphorylate or adenylate these drugs (Table II). The discovery of the inactivating mechanisms allowed to synthesize analogues such as 3',4'-dideoxykanamycin B (DKB) and amikacin (BB-K8) which are superior to their respective starting materials. These analogues are no longer substrates for the inactivating enzymes and therefore inhibit the growth of bacteria resistant to the original aminoglycosides.

With the exception of chloramphenicol acetyltransferase that seems to be intimately associated with the cell membrane, these enzymes (including β -lactamases) are located between the membrane and the cell wall (the periplasm). Their synthesis in the cell is directed by extrachromosomal elements called R-factors.

Table II
Inactivation of chloramphenicol and aminoglycoside antibiotics [67]

Enzyme	Type and site of inactivation	Antibiotics inactivated
Chloramphenicol acetyltransferase	Acetylation of $-\text{CH}_2\text{OH}$ and $-\text{CHOH}$ at C-1 and C-3	Chloramphenicol
Kanamycin acetyltransferase	Acetylation of $-\text{NH}_2$ of an aminohexose at C-6'	Kanamycins A, B, DKB, BB-K8; neomycins B, C; butirosin; ribostamycin; gentamicins C_{1a} , C_2 ; sisomycin; tobramycin
Gentamicin acetyltransferase I	Acetylation of $-\text{NH}_2$ of 2-deoxystreptamine at C-3	Gentamicins C_1 , C_{1a} , C_2 ; sisomycin; tobramycin
Gentamicin acetyltransferase II	Acetylation of $-\text{NH}_2$ of an aminohexose at C-2'	Gentamicins C_1 , C_{1a} , C_2 ; sisomycin
Neomycin-kanamycin phosphotransferase (2 enzymes: I and II)	Phosphorylation of $-\text{OH}$ of an aminohexose at C-3'	Neomycins A, B, C; paromomycin; kanamycins A, B, C; ribostamycin; gentamicin A; lividomycin B. Enzyme II also phosphorylates butirosin
Lividomycin phosphotransferase	Phosphorylation of $-\text{OH}$ of D-ribose at C-5''	Lividomycin B; neomycins B, C; paromomycin; ribostamycin
Streptomycin phosphotransferase	Phosphorylation of $-\text{OH}$ of N-methylglucosamine at C-3	Streptomycins (including bluen-somycin)
Gentamicin adenylyltransferase	Adenylylation of $-\text{OH}$ of aminohexose at C-2''	Gentamicins A, C, C_a , C_c ; sisomycin; tobramycin; kanamycins A, B, C
Streptomycin-spectinomycin adenylyltransferase	Adenylylation of $-\text{OH}$ of N-methylglucosamine at C-3 (streptomycin) and of $-\text{OH}$ of actinamine at C-4 (spectinomycin)	Streptomycins and spectinomycin

The enzyme penicillin amidase carries out the hydrolysis of penicillins whereby 6-aminopenicillanic acid (6-APA) is formed. This reaction has been of singular importance since 6-APA had become instrumental in the generation of novel semisynthetic penicillins. They possess broader antibacterial spectra than benzylpenicillin, can be administered orally, are stable in acid media, and are resistant to β -lactamase. Although 6-APA has been prepared from benzylpenicillin by chemical hydrolysis in 1970 [68], the enzymatic method remains the preferred way of its production. Microbial hydrolysis of novobiocin and bleomycin may also become of practical importance, if useful analogues of these antibiotics are found [cf. 69, 70].

The microorganisms which elaborate this amidase have been investigated extensively also because they carry out the reversal of the hydrolysis, namely the synthesis of penicillin from 6-APA and the suitable side-chains [cf. 71]. Indeed, a microbial method is now available whereby benzylpenicillin can be

converted to ampicillin by proper selection and mutation of suitable bacteria without resorting to the isolation of the intermediary 6-APA [72, 73].

In addition to the β -lactamases and transferases, several enzymes involved in the conversion of antibiotics have been investigated. In some cases they have been induced either by the substrate antibiotics, their analogues, or by portions of their molecules. Those which have been isolated and purified to different degree are listed in Table III.

Table III
Purification of antibiotic-converting enzymes

Antibiotic	Reaction	Source of enzyme	Purification	Reference
Actinomycin	Hydrolysis of lactone	<i>Actinoplanes missouriensis</i>	90-fold	[48]
Cephalosporin	Hydrolysis of lactam	<i>Bacillus cereus</i>	Crystals	[74]
	Hydrolysis of amide	<i>Acetobacter turbidans</i>	48-fold	[75]
Chloramphenicol	Acetylation	<i>Streptococcus epidermidis</i>	171-fold	[13]
		<i>Escherichia coli</i>	41-fold	[14]
		<i>E. coli</i>	17—30-fold	[76]
Dihydroabikoviromycin	Dehydrogenation	<i>Streptomyces</i> sp.	120-fold	[26]
Dihydrostreptomycin	Phosphorylation	<i>Pseudomonas aeruginosa</i>	205-fold	[77]
Dihydrostaphylomycin S	Hydrolysis of lactone	<i>A. missouriensis</i>	200-fold	[48]
Echinomycin				
Etamycin				
Gramicidin S	Hydrolysis of peptide	<i>B. subtilis</i> proteinase	Crystals	[78]
Kanamycin	Phosphorylation	<i>P. aeruginosa</i>	350-fold	[79]
Nisin	Reduction	<i>B. cereus</i>	12—20-fold	[80]
Penicillin	Hydrolysis of lactam	<i>B. cereus</i>	Crystals	[81]
	Hydrolysis of amide	<i>B. megaterium</i>	91-fold	[81]
		<i>E. coli</i>	Crystals	[82]
Streptomycin	Adenylation	<i>E. coli</i>	100-fold	[83]
Streptomycin 6-phosphate	Hydrolysis of phosphate	<i>S. griseus</i>	50-fold	[84]
Subtilin	Reduction	<i>B. cereus</i>	12—20-fold	[80]
Toyocamycin	Hydration	<i>S. rimosus</i>	24-fold	[40]

These reactions were carried out in aqueous solutions, but more recently the techniques of immobilized enzymes [85] have also been applied to these processes. In particular, penicillin amidase has been immobilized by covalent binding to DEAE-cellulose [86] or trapping in yarns of cellulose acetate [87].

Similarly, the immobilization of streptomycin and neomycin-kanamycin phosphotransferases with Sepharose was reported [88] and the applicability of others will undoubtedly be also tested. Some properties of the enzymes may change under such conditions, but not to the detriment of the reaction in question. Their preservation for re-use, extension of their life as well as recovery of products free of undesirable by-products, may greatly increase the economy of such processes.

Microorganisms are extremely valuable tools in the synthesis of certain chemicals. Their enzymes have been used to carry out reactions which are difficult to perform by purely chemical means [89]. They have been employed in those instances in which the modification of only one of several substituents on the substrate molecule is desired. Because of this specificity, the protection of other reactive groups, which is essential for the chemical manipulation, is not needed. In addition, the inclusion of microbial steps in the synthesis of certain chemicals has simplified such syntheses and made their production more economical: L-ascorbic [90] and L-glutamic [91] acids, L-lysine [92], L-ephedrine [93], L-dopa [94], or steroid hormones [9, 95-97].

Similarly, enzymatic reactions of antibiotics can be expected to be used only in those cases in which specificity is required and when they offer a definite advantage over the chemical manipulation. The results obtained thus far with this approach have been disappointing as even small changes in the antibiotic molecule have yielded partially or completely inactive products. The usefulness of 6-APA and discovery of 5-hydroxy-7-chlortetracycline (and 6-demethyltetracyclines), adriamycin or N-demethylclindamycin, should, however, stimulate further work of this kind and intensify the effort which to date has been modest in comparison to massive screening and extensive programmes of chemical modifications.

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MICROBIAL DEGRADATION OF STEROID ALKALOIDS.

EFFECT OF NITROGEN ATOM IN THE SIDE-CHAIN ON THE MICROBIAL DEGRADATION OF STEROID ALKALOIDS

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Summary. The microbial dehydrogenation of steroid alkaloids follows the dehydrogenation pattern of steroids until the 3-keto-1,4-diene stage. No side-chain cleavage or degradation of the steroid nucleus is observed. Side-chain cleavage of tomatidine is achieved only by previous induction of side-chain splitting enzymes.

In the microbial transformations of steroids a known process is their degradation. The initial steps of this degradation, shown in Fig. 1, occur in ring A. Starting with 3-ol steroids, a 3-keto compound is first obtained, which is dehydrogenated to 3-keto-4-ene or 3-keto-1-ene compounds and finally to a 3-keto-1,4-diene compound. The next step is a 9 α -hydroxylation which leads

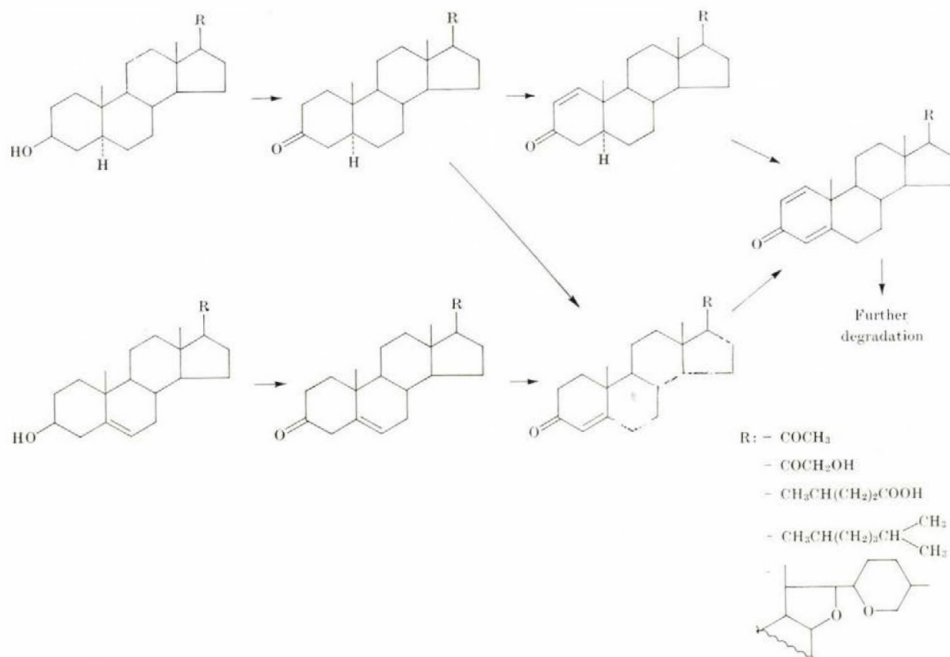


Fig. 1. Microbial dehydrogenation of steroids

to the opening of the ring B and subsequent production of smaller fragments and eventually to a breakdown to CO_2 and H_2O . The side-chain at C17 is usually split off simultaneously and in this way 1,4-androstadiene-3,17-dione is formed. In such cases this is the main intermediate before the breakdown of the steroid molecule.

Our research into the microbial degradation of steroid alkaloids started with tomatidine as the alkaloid.* *Fusarium solani* was chosen as the microorganism, since it can transform progesterone into 1,4-androstadiene-3,17-dione [1]. We have achieved this transformation with yields over 90% [2]. KONDO and MITSUGI [3] reported that diosgenin is transformed by *Fusarium solani* into 1,4-androstadiene-3,16-dione. We found, however, that neither *F. solani* nor other *Fusarium* strains transformed tomatidine [4]. We have therefore tried other microorganisms known to be good steroid degraders such as *Nocardia* and *Mycobacterium*, and found that *Nocardia restrictus* and *Mycobacterium phlei* do indeed transform tomatidine. Since no essential differences were found between those strains, only the transformation with *N. restrictus* was examined in detail [5].

It was established that tomatidine (I) is dehydrogenated first to tomatanin-3-one (II), then double bonds 4 : 5 (4-tomatenin-3-one, III) and 1 : 2

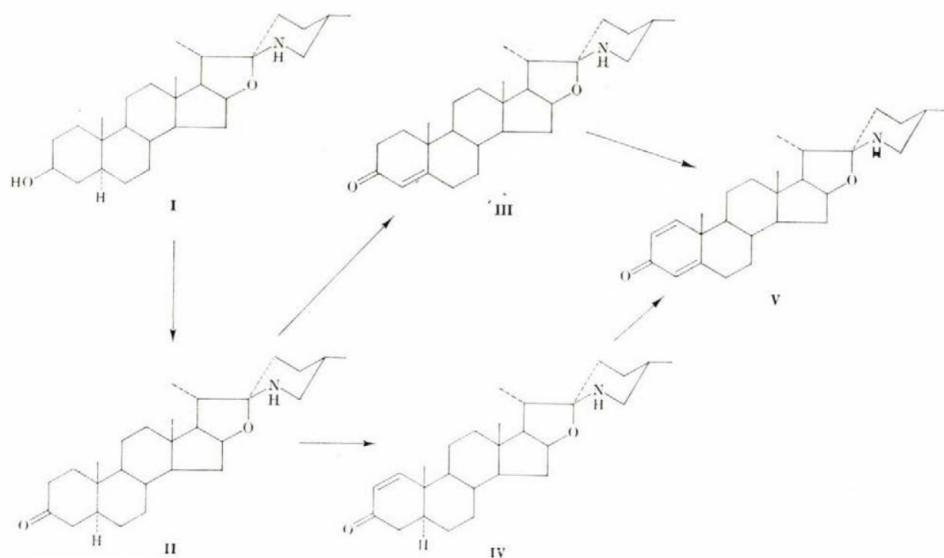


Fig. 2. Dehydrogenation of tomatidine by *N. restrictus*

* IUPAC nomenclature: tomatidine = (22S, 25S)-5 α -tomatanin-3 β -ol; 5 α -solasodanol = (22R, 25R)-5 α -tomatanin-3 β -ol; 1,4-solasodadien-3-one = (22R, 25R)-1,4-tomatadien-3-one; 1,4-tomatadien-3-one = (22S, 25S)-1,4-tomatadien-3-one.

(1-tomatenin-3-one, IV) are introduced and finally 1,4-tomatadiene-3-one (V) is obtained as shown in Fig. 2. The dehydrogenation pattern known to operate in steroids (see Fig. 1) is, therefore, followed until the 3-keto-1,4-diene stage. But no side-chain cleavage or further degradation of the steroid nucleus of tomatidine could be observed, in spite of efforts spent to achieve this goal by changing the experimental conditions.

The course of the transformations of tomatidine was followed by thin-layer chromatography. For the identification of the metabolites we relied mainly on mass spectrometry since steroid alkaloids give very characteristic mass spectra. As an example the mass spectra of tomatidine (I) and 1,4-tomatadiene-3-one (V) are shown in Fig. 3. The mass spectrum of the metabolite shows the molecular ion shifted to a mass value six units lower, meaning that six hydrogen atoms have been lost. The peak at m/e 121 indicates the 1,4-diene-3-keto structure of ring A, and peaks at m/e 114 and 138 confirm the unchanged structure of rings E and F of tomatidine [6]. From the mass spectrum alone, therefore, the structure of the metabolite can be deduced. Of course, other spectroscopic methods additionally support the deduced structure.

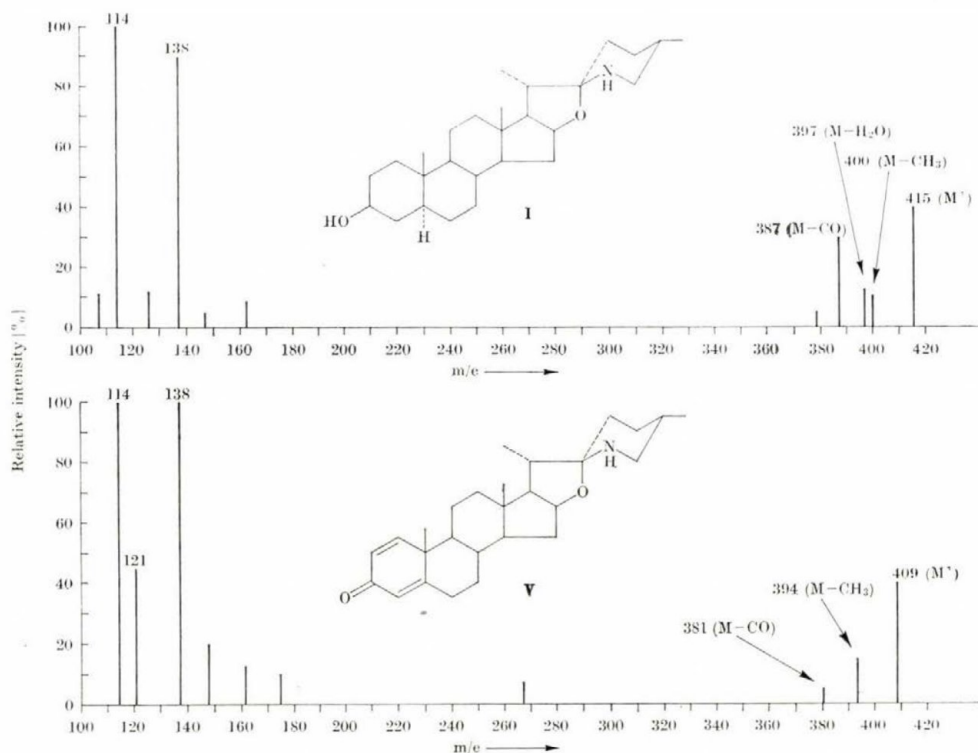


Fig. 3. Mass spectra of tomatidine and 1,4-tomatadiene-3-one

We were next interested to learn more about the structural requirements of such a steroid alkaloid which would show the same specific pattern of degradation as described above for tomatidine. When (22R, 25R)-5 α -solasodanol (VI), differing from (22S, 25S)-5 α -tomatidine only in the configuration of ring F, was incubated with *N. restrictus*, 1,4-solasodadiene-3-one (VII) was obtained as presented in Fig. 4, although the yield was lower than in the case of tomatidine [7]. Therefore, the stereochemistry of ring F seems to have little effect on the course of the dehydrogenation of tomatidine.

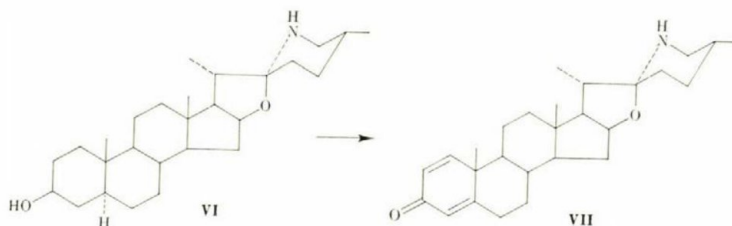


Fig. 4. Dehydrogenation of 5 α -solasodanol by *N. restrictus*

It was assumed that the cyclic structure of the side-chain of tomatidine might affect the course of the degradation. To find out whether changes in the side-chain of tomatidine could have some effect, we prepared dihydrotomatidines with an open ring E by reduction of tomatidine according to the method of ADAM and SCHREIBER [8]. When these compounds (22S, 25S)-22,26-epimino-5 α -cholestane-3 β ,16 β -diol (VIII) and (22R, 25S)-22,26-epimino-5 α -cholestane-3 β ,16 β -diol (X) were incubated with *N. restrictus*, the same results as with tomatidine were obtained. Both were dehydrogenated in high yield to the corresponding 22,26-epimino-1,4-cholestadien-3-ones IX and XI, respectively (Fig. 5) and no further transformation could be observed [9].

The dihydrotomatidine with an opened ring F in the form of its amino-acetate (20S, 22 ξ , 25 ξ)-26-acetyl-amino-5 α -furostan-3 β -ol (XII), was prepared according to SATO and LATHAM [10]. Some incubations of this compound yielded the expected 1,4-diene-3-keto derivative (20S, 22 ξ , 25 ξ)-26-acetyl-amino-1,4-furostadien-3-one (XIII), even though the yield was very low, while other incubations yielded no 1,4-diene at all. As this behaviour might have been due to the presence of the acetylated amino group in the side-chain, incubation with the deacetylated compound seemed desirable. The deacetylated compound, (25 ξ)-26-amino-5 α -cholestane-3 β , 16 β ,22 ξ -triol (XV) was prepared by refluxing XII with 6 N hydrochloric acid, but compound XV thus prepared also had an opened E ring. Incubation of this compound produced only one detectable metabolite which could be identified by its mass spectrum and comparison with the intermediate XIV obtained in the hydrolysis of

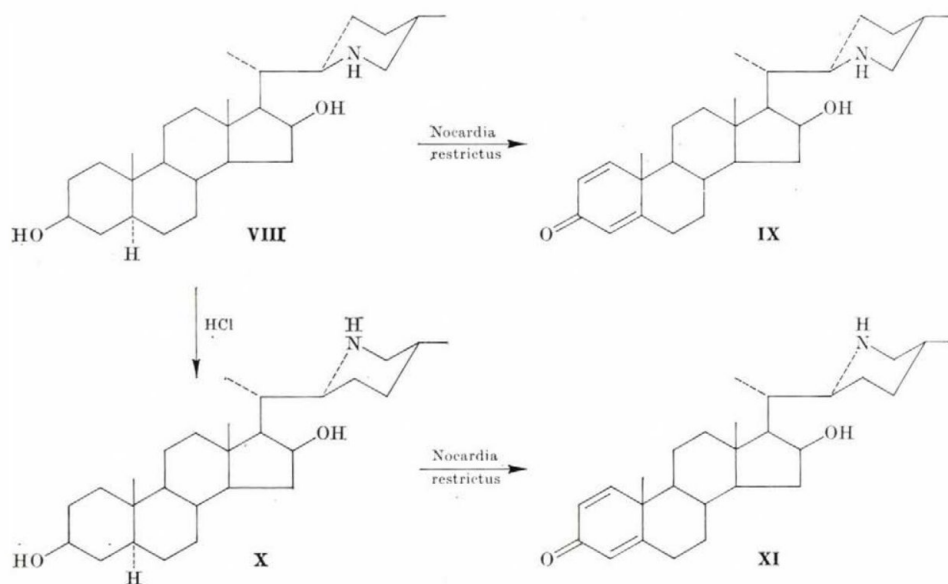


Fig. 5. Dehydrogenation of dihydrotomatidines with open ring E by *N. restrictus*

XII as (25 ξ)-26-acetylamino-5 α -cholestane-3 β ,16 β ,22 ξ -triol (XIV). Fig. 6 shows the transformations.

The microbial acetylation of an amino group in the side-chain of steroid was first reported by SMITH *et al.* [11]. They found that *Streptomyces roseochromogenus* acetylates 21-amino-9 α -fluoro-11 β ,17 α -dihydroxy-4-pregnene-3,20-dione to its 21-acetylamino derivative.

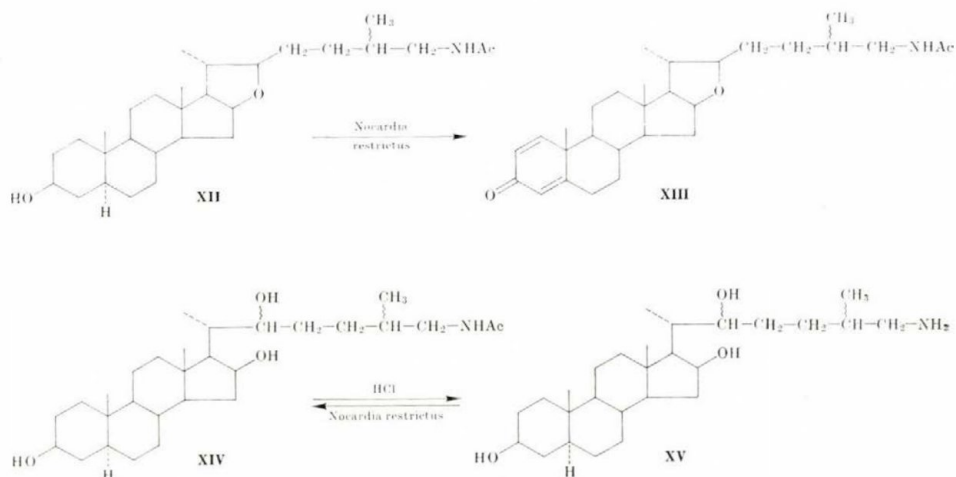


Fig. 6. Dehydrogenation of dihydrotomatidine with open ring F by *N. restrictus*

These results indicate that the presence of a nitrogen atom in the side-chain of a steroid alkaloid has an effect on the course of microbial transformation. If an aliphatic amino group is present in the side-chain of a steroid, acetylation takes place. If the nitrogen atom is heterocyclic, dehydrogenations in ring A proceed normally as known to occur in steroids, but further degradation of the steroid nucleus beyond the 1,4-diene-3-keto stage is prevented and no cleavage of the side-chain occurs. This behaviour differs from the behaviour of e.g. sapogenins, which contain an oxygen atom instead of nitrogen in the side-chain, and which *Mycobacterium phlei* transforms into 1,4-androstadiene-3,17-dione, according to AMBRUS and BÜKI [12]. On the other hand, HOWE *et al.* [13] found only metabolites with an intact side-chain when diosgenin and hecogenin were incubated with an unidentified bacterium (ATCC 3660) and *Nocardia globerula*.

The presence of a nitrogen atom in ring C seems to have the same effect as the nitrogen in the side-chain. MAZUR and MUIR [14] described the dehydrogenation of 12a-aza-3 β ,17 α -dihydroxy-C-homo-5 α -pregnane-12,20-dione into 12a-aza-17 α -hydroxy-C-homo-1,4-pregnadiene-3,12,20-trione by a *Nocardia* sp.

Two explanations can be envisaged of the described specific behaviour of the steroid alkaloids: they do not act as inducers for the side-chain splitting enzymes or they are not substrates for such enzymes. We tested these possibilities by inducing the side-chain splitting enzymes with cholesterol in *Arthrobacter simplex* and then incubated tomatidine in the presence of α,α -dipyridyl to prevent the breakdown of the steroid nucleus [15]. In this way tomatidine was transformed to 1,4-androstadiene-3,17-dione in 2.4–4.5% yield. Although this yield was lower than that of 15% obtained by NAGASAWA *et al.* [16] with cholesterol and *A. simplex*, it compares favourably with the 4 and 2.5% yields obtained by AMBRUS and BÜKI [12] from tigogenin and neotigogenin, respectively, with *M. phlei*.

Thus, the nitrogen atom seems to prevent the induction of the side-chain splitting enzymes. If they are induced by some suitable steroid in a suitable microorganism, the side-chain of tomatidine is cleaved as happens in other steroids not having a nitrogen atom in the side-chain.

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DIE VERWENDUNG VON MIKROBIELLEN ENZYMEN BEI STOFFWECHSELSTUDIEN DER STEROIDE

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J. SCHLEGEL und K. SCHUBERT (*Institute of Microbiology and Experimental Therapy, Academy of Sciences, Jena, GDR*): **Application of microbial enzymes in studies of steroid metabolism.** *In vitro* models are necessary for studying the biotransformation of new steroid drugs and to determine the structure of the metabolites and their biological activity. For that reason microorganisms and their enzymes were used to investigate the anabolic steroid Oral-Turinabol® (4-chloro-17 α -methyl-17 β -hydroxy-1,4-androstadiene-3-one). *Clostridium paraputrificum* transformed Oral-Turinabol® into the hydrogenation products 4 β -chloro-17 α -methyl-17 β -hydroxy-5 β -1-androstene-3-one (I) and 4 β -chloro-17 α -methyl-5 β -1-androstene-3 α ,17 β -diol (II); *Rhodotorula glutinis* to 4 α -chloro-17 α -methyl-17 β -hydroxy-5 α -1-androstene-3-one (III) and 4 α -chloro-17 α -methyl-5 α -1-androstene-3 β ,17 β -diol (IV).

The hydroxylation products 6 β -hydroxy- (V), 7 β -hydroxy- (VI), 15 α -hydroxy- (VII) and 15 β -hydroxy-Oral-Turinabol (VIII) resulted from *Absidia glauca* and *Aspergillus flavus*. The metabolites II—V were isolated until now from mammals.

Zusammenfassung. Es wird angestrebt, *in vitro* Modell-Versuche durchzuführen, damit die Biotransformation neuer Steroid-Pharmaka, die Struktur der Metaboliten und deren biologische Wirkungen bestimmt werden können. Zu diesem Zweck wurden Mikroorganismen und deren Enzyme zur Untersuchung des anabol wirksamen Steroids Oral-Turinabol® (4-Chlor-17 α -methyl-17 β -hydroxy-1,4-androstadien-3-on) verwendet. Oral-Turinabol® wurde durch *Clostridium paraputrificum* in 2 Hydrierungsprodukte, 4 β -Chlor-17 α -methyl-17 β -hydroxy-5 β -1-androsten-3-on (I) und 4 β -Chlor-17 α -methyl-5 β -1-androsten-3 α ,17 β -diol (II), umgewandelt. *Rhodotorula glutinis* bildete 4 α -Chlor-17 α -methyl-17 β -hydroxy-5 α -1-androsten-3-on (III) und 4 α -Chlor-17 α -methyl-5 α -1-androsten-3 β ,17 β -diol (IV). Als hydroxylierte Produkte wurden 6 β -Hydroxy-, 7 β -Hydroxy-, 15 α -Hydroxy- und 15 β -Hydroxy-Derivate (V—VIII) von Oral-Turinabol® mit einem *Absidia glauca* und mit einem *Aspergillus flavus* Stamm erhalten. Die Metaboliten II—V sind bisher aus Säugetieren isoliert worden.

Die Modifizierung des Steroidmoleküls zu einem Pharmakon wird durch Einführung zusätzlicher Substituenten bzw. Strukturparameter erreicht. Bei der Anwendung solcher neuer, strukturell und in der Wirkung differenzierter Steroidpharmaka sind Änderungen in der Metabolisierung zu erwarten. Beispielsweise verursachen Substituenten in bestimmten Positionen von Steroidhormonen eine Verlangsamung des Stoffwechsels dieser Pharmaka. Da der Organismus über Alternativwege in der Metabolisierung von Steroiden verfügt, treten nicht nur in quantitativer sondern auch in qualitativer Hinsicht Verschiebungen auf. So kann der Hauptumwandlungsweg bei endogenen Steroiden — nämlich die Hydrierung — im Falle bestimmter Steroidpharmaka zugunsten der Hydroxylierung zurücktreten.

Bei der Untersuchung neuer Steroidwirkstoffe können nicht in jedem Falle ausgedehnte Stoffwechselstudien durchgeführt werden, deren Hauptproblem die Isolierung und Charakterisierung der Metaboliten darstellt. Es wird daher angestrebt, mit Hilfe einfacher biochemischer Modelle Aufschluß über die Biotransformation dieser Verbindungen zu erhalten. Teilaspekte der Metabolisierung können dann in gezielten *in vivo* Studien weiter bearbeitet werden.

Mit der Bildung von Metaboliten stellt sich die Frage nach deren Partialwirkung. Im Gegensatz zu früheren Anschauungen werden Steroidmetaboliten nicht nur als Inaktivierungsprodukte angesehen. Ein für die Zukunft bedeutender Aspekt liegt in der Auffindung von Wirkungen, die von Steroidmetaboliten ausgehen. Eine der wesentlichsten Voraussetzungen hierfür ist die Bereitstellung ausreichender Substanzmengen.

Bei *in vivo* Stoffwechseluntersuchungen werden im günstigen Fall nur wenige Milligramm eines Metaboliten isoliert, dessen chemische Synthese aufgrund der komplexen Struktur des Pharmakons oft nicht möglich ist. Es bedarf besonderer Anstrengungen, um über ausreichende Substanzmengen verfügen zu können.

Beide Probleme, Kenntnis der Biotransformationshauptwege sowie Gewinnung von Metaboliten des Pharmakons im präparativen Maßstab können durch den Einsatz von ausgewählten Mikroorganismen bzw. deren steroidtransformierenden Enzymen einer Lösung zugeführt werden. Am Beispiel des anabol wirksamen Steroids Oral-Turinabol® (Jenapharm, Jena, DDR) [1–3] soll dieser Sachverhalt kurz skizziert werden. Oral-Turinabol® besitzt folgende Struktur (Abb. 1).

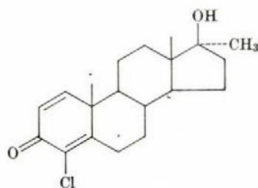


Abb. 1. Oral-Turinabol®. Jenapharm, Jena, DDR

Als besonderes Strukturmerkmal ist der Chlorsubstituent in 4-Stellung in Verbindung mit der 1,4-Doppelbindung anzusehen. Aus Stoffwechseluntersuchungen beim Menschen [4, 5] ging hervor, daß die Hydrierung des Ringes A anscheinend zugunsten des hydroxylierenden Stoffwechsels zurücktritt. Aus einer Anzahl von Metaboliten konnten die 6 β - und 16 β -Hydroxy- sowie die 6 β -, 16 β -Dihydroxyverbindungen, aber keine der erwarteten Hydrierungsprodukte identifiziert werden.

In Verbindung mit den *in vivo* Studien wurde damit begonnen, die Metabolisierung von Oral-Turinabol® durch Einsatz mikrobieller Modelle zu untersuchen.

In unserer Abteilung sind in den vergangenen Jahren auf mikrobieller Basis enzymatische Reaktionen aufgefunden worden, die die Steroidbiotransformation im Säugetierorganismus betreffen. Mit einer größeren Anzahl von Steroiden wurde der stereospezifische Verlauf dieser Reaktionen überprüft. Dies betrifft insbesondere die Hydrierung des Ringes A und B ungesättigter 3-Ketosteroiden sowie die Hydroxylierung in verschiedenen Positionen [6–9].

Vom Oral-Turinabol® wurden 2 Hydrierungsprodukte mit *Clostridium paraputrificum* erhalten (Abb. 2).

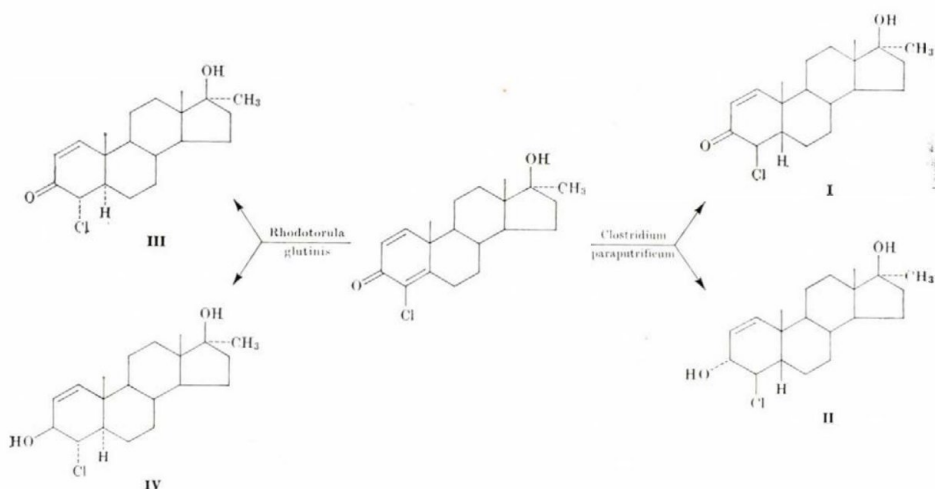


Abb. 2. Mikrobielle Hydrierung von Oral-Turinabol®

In beiden Fällen handelt es sich um Metaboliten vom 5β-Typ, in denen das H-Atom am C-5 Atom die β-Konfiguration besitzt. Die Hydrierung durch Hydrogenasen dieser *Clostridium*-Spezies verläuft streng stereospezifisch. Bei etwa dreißig 3-Ketosteroiden mit unterschiedlicher Anzahl Doppelbindungen im Ring A bzw. A und B wurden jeweils Metaboliten vom 5β-Typ mit einer 3α-Hydroxygruppe gebildet. Ausbeuten zwischen 30–90% wurden erzielt. Im Gegensatz zu anderen 1,4-Dienen wurde bei Oral-Turinabol® die 1-Doppelbindung nicht reduziert.

Weitere 2 Hydrierungsprodukte jedoch vom 5α-Typ wurden mit *Rhodotorula glutinis* gewonnen (Abb. 2). Der 5α-H/3β-OH Metabolit wird in Ausbeuten von 65% erhalten. Das 3-Keton tritt nur in geringen Mengen auf, kann aber leicht durch Chromsäureoxidation nach Jones aus dem 3-Hydroxyderivat erhalten werden. Auch hier wurde die Stereospezifität der mikrobiell

enzymatischen Reaktion mit verschiedenen Ring A ungesättigten 3-Ketosteroiden überprüft. Die 5 α -Hydrierung wurde in allen Fällen beobachtet.

Im Gegensatz zu Oral-Turinabol®[®], bei dem nur der 3 β -Hydroxymetabolit entsteht, wurden Metaboliten mit der 3 α - und 3 β -Hydroxygruppe im Verhältnis von etwa 5 : 4 gebildet.

Bei der Hydrierung durch beide Spezies fällt weiterhin auf, daß die 1-Doppelbindung von Oral-Turinabol®[®] nicht angegriffen wird. Dies läßt ein charakteristisches Verhalten des Pharmakons enzymatischen Reduktionen gegenüber erkennen. Weiterhin kann auf ein besonderes Stoffwechselverhalten im Säugetierorganismus geschlossen werden.

Die insgesamt erschwerte Hydrierung von Oral-Turinabol®[®] im mikrobiellen Modell bekräftigte einerseits die Ergebnisse und Schlußfolgerungen der eingangs zitierten Stoffwechselstudie [4, 5]. Bei dieser wurden nur hydroxylierte Metaboliten gefunden und UV- und UR-spektrographische Daten wiesen weitere, nicht genau identifizierte Metaboliten mit unverändertem Ring A auf.

Andererseits legte die Transformation von Oral-Turinabol®[®] zu bestimmten Hydrierungsprodukten auf mikrobiell enzymatischem Wege auch deren Bildung im Säugetierorganismus nahe (Abb. 3).

Dies war Anlaß, gezielte Stoffwechseluntersuchungen erst einmal bei Versuchstieren wie Affe und Ratte durchzuführen. Durch diese Versuche wurde

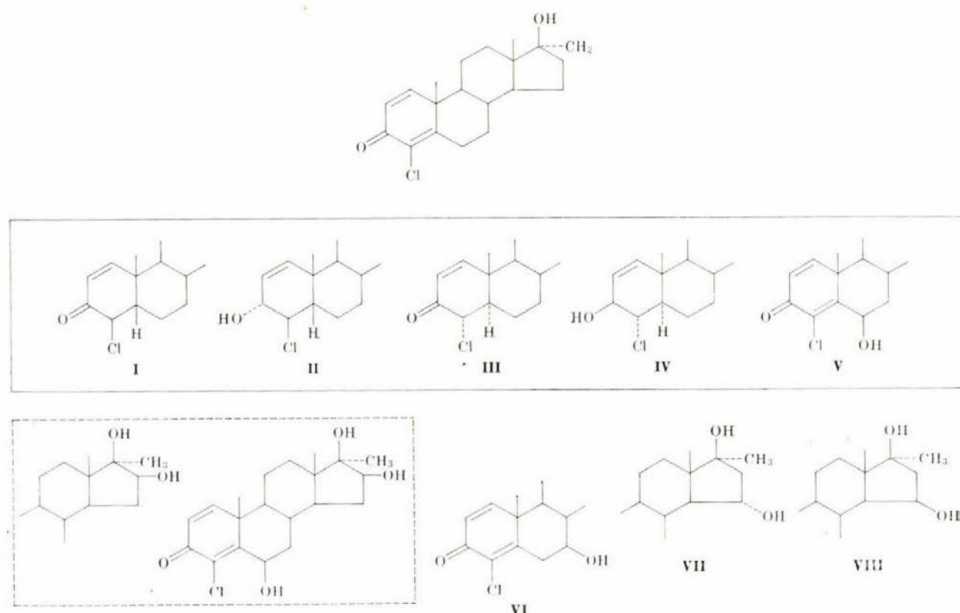


Abb. 3. Metaboliten von Oral-Turinabol®[®] aus Säugetierorganismus und Mikroorganismen; aus Säugetierorganismus; ohne besonderes Kennzeichnen: aus Mikroorganismen

der Nachweis erbracht, daß Hydrierungsprodukte im Säugetier gebildet werden.

Beim Pavian entstehen beide auf mikrobiellem Wege erhaltene Metaboliten vom 5β -Typ und bei der Ratte die zwei Metaboliten vom 5α -Typ. Weiterhin wurde in geringen Mengen ein 3α -Hydroxymetabolit vom 5α -Typ festgestellt.

Durch den Einsatz weiterer mikrobieller Modelle wurde auch eine Anzahl hydroxylierter Stoffwechselprodukte von Oral-Turinabol® gewonnen, die für die Charakterisierung der Metaboliten in der eingangs erwähnten Stoffwechselstudie bedeutsam waren (Abb. 4).

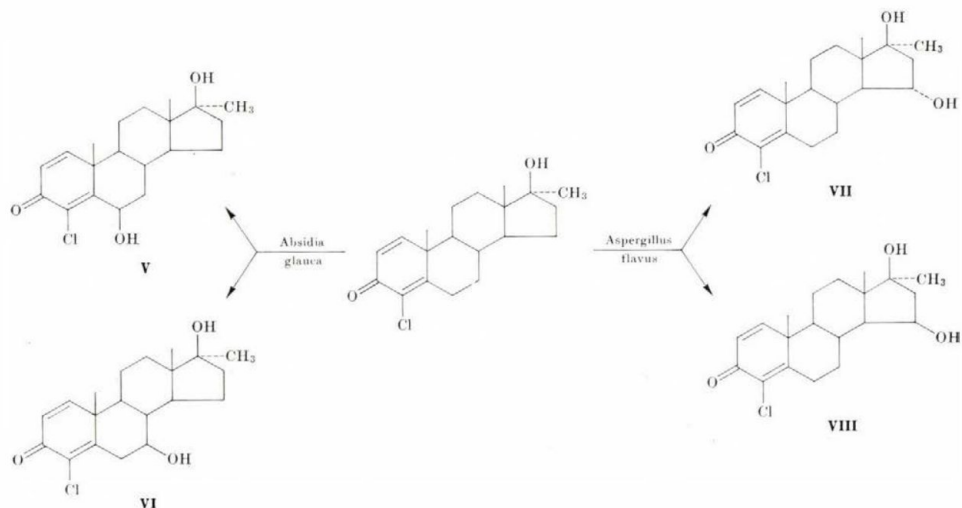


Abb. 4. Mikrobielle Hydroxylierung von Oral-Turinabol®

Mit einem *Absidia glauca* Stamm konnte der 6β - und 7β -Hydroxymetabolit von Oral-Turinabol® erhalten werden. In 15-Stellung hydroxyliertes Oral-Turinabol® wurde mit *Aspergillus flavus* hergestellt. Als besonderes Charakteristikum dieser Reaktion ist die gleichzeitige Einführung einer 15α - und 15β -Hydroxygruppe anzusehen. Die mikrobielle Transformation von Oral-Turinabol® erfolgte in der üblichen Weise durch Zugabe der Substanz zu den Kulturmedien und anschließende aerobe bzw. im Falle von *Cl. paraputrificum* anaerobe Fermentation. Aus den Chloroform- oder Ätherextrakten der Kulturmedien wurden die Metaboliten auf Aluminiumoxid oder Kieselgelschichten im präparativen Maßstab abgetrennt. Als geeignete Laufmittel erwiesen sich Chloroform-Essigester und Methylenchlorid-Essigester-Gemische sowie mit Wasser gesättigtes Methylenchlorid.

Obwohl durch den Einsatz von Mikroorganismen mit bekannter Stereospezifität der Enzyme Hydrierungsprodukte vom 5 β -H/3 α -OH- und 5 α -H/3 α -OH- bzw. 5 α -H/3 β -OH-Typ erwartet werden konnten, war eine genaue Bestimmung der räumlichen Anordnung des 4-Chloratoms erforderlich, bzw. dessen Einfluß auf die bisher beobachtete Stereospezifität der Hydrierung.

Durch physikalische und chemische Verfahren sowie durch Überführung der Metaboliten in bekannte Verbindungen und deren Vergleich wurden die angegebenen Konfigurationen ermittelt bzw. bestätigt [10]. Die Metaboliten wurden im g-Maßstab hergestellt. Außer einer genauen Konfigurationsermittlung erlaubte dies auch die tierexperimentelle Testung der biologischen Aktivität und biochemische Untersuchung der Metaboliten. Die Versuche sind noch nicht abgeschlossen. Bisher wurde festgestellt, daß der Metabolit III, das 5 α -Dihydroderivat, stärker anabol wirksam ist als Oral-Turinabol® und auch eine günstige androgen/anabole Wirkungsdissoziation besitzt. Die 5 β -Derivate sind wie erwartet anabol/androgen unwirksam (Abb. 3).

Zusammenfassend läßt sich feststellen: Durch den Einsatz von Modelluntersuchungen mit ausgewählten Mikroorganismen bzw. deren Enzymen in Kombination mit gezielten *in vivo*-Studien ist es gelungen, wesentliche und neue Aspekte des Stoffwechsels von Oral-Turinabol® aufzuklären. Auf mikrobieller Basis konnten Metaboliten im g-Maßstab gewonnen werden, so daß deren tierexperimentelle Testung möglich wurde. Metabolit III besitzt gegenüber Oral-Turinabol® eine stärkere anabole Wirkung. Damit ergeben sich Hinweise zum Wirkungsmechanismus dieses Pharmakons.

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THE MECHANISM OF ACTION OF GLYCOSIDASES

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Summary. The factors that may contribute to the rate enhancement observed with enzymatic versus non-enzymatic hydrolysis of glycosides are discussed. The nature of the active site as deduced from labelling studies with β -glucosidases is described. A two-step mechanism involving either an enzyme stabilized glycosyl ion or a covalent glycosyl-enzyme intermediate is proposed. Experiments with a β -glucosidase from almonds show that even with 2-deoxy glucosides with good leaving groups as aglycon which are hydrolyzed 1000 times more slowly than the corresponding glucosides, the deglycosylation step is faster than the cleavage of the glycosidic bond.

Non-enzymatic hydrolysis of glycosides is catalyzed by acids and proceeds by an A1 mechanism (Fig. 1). The overall rate is determined by the equilibrium

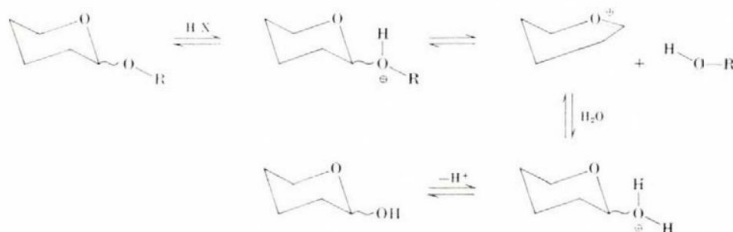


Fig. 1. Hydrolysis of glycosides catalyzed by acids

concentration of the protonated glycoside and by the unimolecular formation of the glycosyl ion by cleavage of the O-glycosyl bond. Electronic effects in the aglycon act in opposite directions on the equilibrium and bond cleavage steps, and cancel each other to a considerable extent. A considerable influence is, however, exerted by inductive effects of substituents in the pyranoid ring which influence the basicity and ease of formation of the carbonium ion in a concerted manner. This results in a rate increase of more than 10^6 from a glucoside to a tetrahydropyranyl ether (Fig. 2).

Catalysis by bases is observed only with glycosides containing a good leaving group like aryl or enol glycosides. The reaction proceeds by an intramolecular nucleophilic displacement reaction (Fig. 3).

The acid HX necessary for acid catalysis is the hydronium ion. No general acid catalysis has been observed with glycosides. General acid catalysis does,

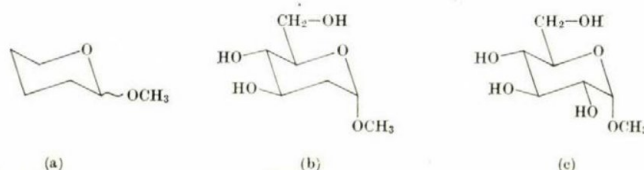


Fig. 2. Relative rates of hydrolysis at 40°C and $H_0 = 0.4$ [1]. (a) 2-Methoxytetrahydropyran 1 000 000; (b) methyl-2-deoxy- α -glucopyranoside 346; (c) methyl- α -glucopyranoside 0.32

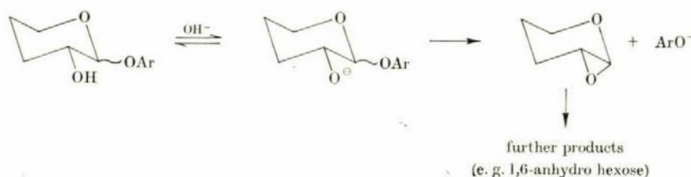


Fig. 3. Hydrolysis by an intramolecular nucleophilic displacement reaction

however, occur with glycosidases. The acids available here are the carboxyl groups of aspartic and glutamic acid, the protonated imidazol of histidine and in some cases the sulfhydryl group of cysteine. Even the first of these is too weak an acid to donate a proton to the glycosyl oxygen in non-enzymatic reactions. If we compare enzymatic and non-enzymatic hydrolysis with these facts in mind we will get equal amounts of glycoside hydrolyzed in 1 second and 10⁵ years (with equimolar amounts of catalyst) and 1 second and 100 years if we take equal weights. The former corresponds to a rate enhancement of 10¹².

What factors then contribute to this high efficiency of nature's catalysts? An important one is certainly a favourable orientation of functional groups in the enzyme substrate complex. A model for this is *o*-carboxyphenyl- β -glucoside (Fig. 4) which is hydrolyzed almost 10⁴ times more rapidly than the *para* derivative [2]. This rate enhancement may be due to intramolecular general acid catalysis or to an increase in oxygen basicity due to the adjacent carboxylate ion. No kinetic distinction can be made between the two possibilities, but both may play a role in enzymatic catalysis. A possible loss of rotational freedom in the enzyme substrate complex would further enhance this effect.

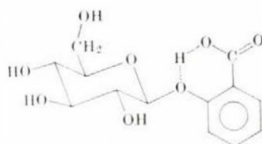


Fig. 4. *o*-Carboxyphenyl- β -glucoside

Another factor to be considered is a steric strain to enforce a conformation that approaches that of the transition state. If the necessary energy would come from the binding process this would result in a considerable reduction of

the energy of activation. Reported values for ΔH^* are in the range of 12 to 16 kcal/mole for enzymatic [3-5] and from 25 to 33 kcal/mole for acid hydrolysis [6] of glycosides. Neglecting differences in the entropy change ΔS^* we can calculate a rate enhancement of 4×10^{10} for a difference in ΔH^* of 15 kcal/mole. The strong competitive inhibition of most glycosidases by hexono- δ -lactones may be cited as experimental support. These lactones have a plane trigonal conformation at C-1 and are in this respect similar to the glycosyl ion. They are usually bound by the active site 10^3 to 10^4 times better than the corresponding free hexoses.

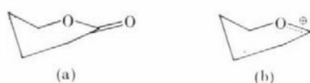


Fig. 5. δ -Lactone (a); pyranosyl ion (b)

More direct evidence for the strain effect is provided by the X-ray structure analysis of lysozyme [7]. This showed that the space available at the bond breaking site will accommodate the pyranose ring of the substrate only if it is deformed in the direction of the carbonium ion conformation.

Similar investigations have not been carried out with other glycosidases. We therefore have to turn to more indirect methods like kinetic studies and labelling of functional groups at the active site. If we combine the results of as many different approaches as possible we may propose a mechanism that will with some luck, bear a resemblance to the actual process.

To label the active site of β -glucosidases we have used epoxides that are structural analogues of the substrate. Their specificity and high reactivity with these enzymes is probably due to the presence of an acid at the active site. This protonates the epoxide oxygen and forms a species that will easily alkylate a nucleophile in a suitable position at the active site. Accordingly the pH-rate profile of the inactivation rate resembles a titration curve decreasing with increasing pH. Its position is similar to the alkaline branch of the pH-profile of $V_{\max}$, pointing to the participation of this group in the catalytic process. With enzymes from *Aspergillus wentii* and from sweet almonds we could identify the nucleophile as carboxylate ion that had formed an ester with the inhibitor conduritol B epoxide [8, 9].

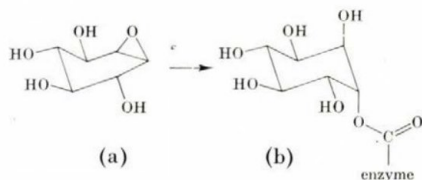


Fig. 6. Inactivation of enzyme by conduritol B epoxide. Conduritol B epoxide (a); ester of (+)-chiro-inositol (b). Incorporation of 1 mole epoxide per mole of enzyme caused total loss of activity

In the aspergillus enzyme the acid involved in the protonation of the substrate and probably of the epoxide too, was identified as carboxyl group by measuring its heat of ionization. The carboxylate ion belongs to an aspartic acid in the following sequence [10]: Val-Met-Ser-*Asp*-Trp-Ala-Ala-His-His-Ala-Gly-Val-Ser-Gly-Ala-Leu-.

From these results we may tentatively propose the following mechanism for the hydrolysis of β -glucosides (Fig. 7). The observed retention of con-

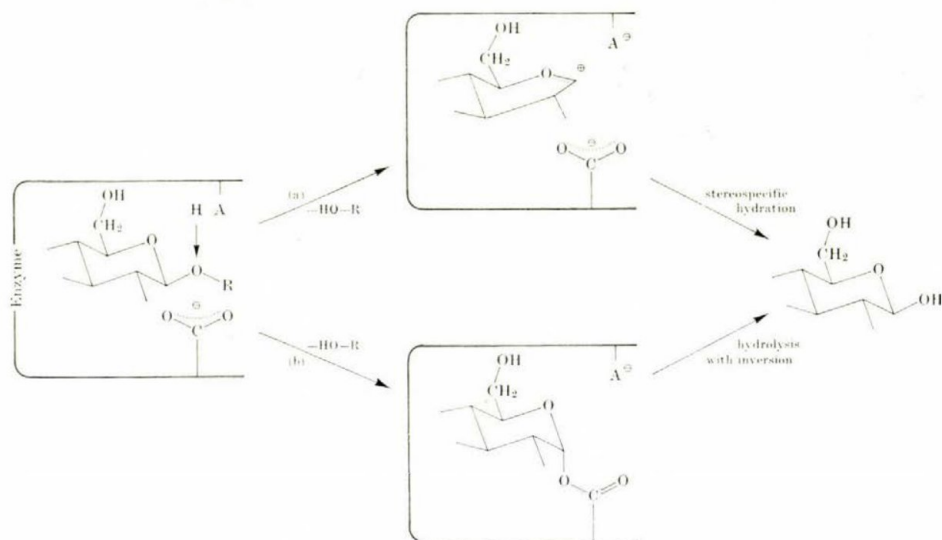


Fig. 7. Proposed mechanism of β -glucoside hydrolysis

figuration at C-1 would either result from a stereospecific addition of water to the carbonium ion or by an acid catalyzed hydrolysis of the ester bond with double inversion. The latter would have to proceed via the formation of a carbonium ion to give the retention of configuration. This type of cleavage is normally observed only with esters of tertiary or benzylic alcohols under markedly acid conditions. Steric strain, however, at the glucosyl binding site might favour the formation of a glucosyl ion to such an extent that this type of hydrolysis occurs here too. A base-catalyzed hydrolysis of the ester bond appears unlikely, because here the normal course is an attack of water or hydroxyl ion at the carbonyl carbon. This, however, would not ensure the desired inversion.

An indication that a covalent intermediate might be formed as shown in pathway (b) comes from two observations. NATH and RYDON [11] observed that with almond β -glucosidase the influence of substituents in phenyl- β -glucosides on $V_{\max}$ was similar to that of their alkaline cleavage which proceeds by a nucleophilic displacement mechanism. The other piece of evidence was

provided by DAHLQUIST *et al.* [12] who measured the secondary isotope effect on the hydrolysis rate of glycosides deuterated at C-1. The effect with lysozyme was equal to that of the acid catalyzed reaction (via a carbonium ion). The isotope effect measured with almond β -glucosidase corresponded to that of the base-catalyzed cleavage.

In order to obtain more direct evidence of a covalent intermediate, we investigated the activity of almond glucosidase A with glycosides containing a good leaving group in which the sugar was modified to ensure a slow hydrolysis. If pathway (b) was operating, the second step might become rate determining and one would then be able to observe a burst in the release of phenol (Fig. 8).

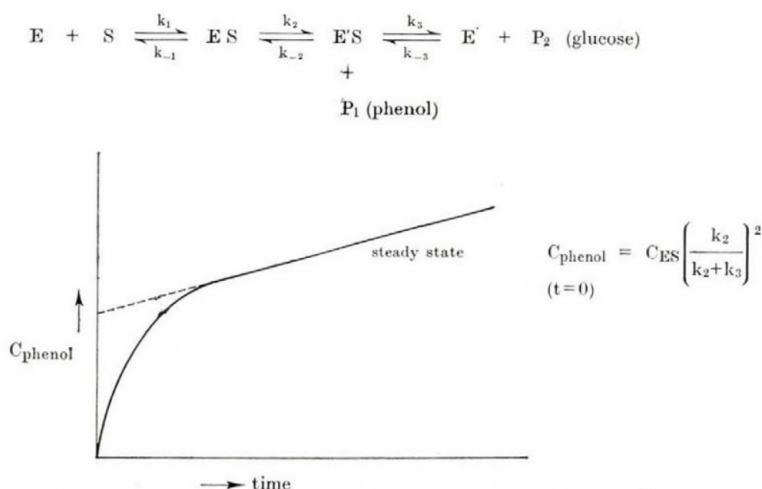


Fig. 8. Influence of phenol release on the rate of reaction pathway

The enzymatic constants of the substrates investigated are given in Table I. Both the xyloside and the 2-deoxyglucoside react at the same site of the enzyme as the glucoside does. Their competitive inhibition constants against p-nitrophenyl β -glucoside agree well with their Michaelis constants. In spite of the high affinity of these substrates to the enzyme, the missing hydroxymethyl group at C-5 or hydroxyl group at C-2 prevents an efficient hydrolysis. The reason for this might be an increased rotational freedom of the sugar part, preventing its correct alignment with respect to the catalytic site or an inability of these substrates to bring the catalytic groups into the correct position by an induced fit mechanism.

With the 2-deoxyglucosides the rate was sufficiently slow to allow measurements with the required high concentrations of enzyme and substrate without having to use a stopped-flow apparatus. The observable burst of free umbelliferon was less than 5% of the expected value. This means that

even with this substrate $k_2 \ll k_3$. Support for this comes from the large influence of the aglycon on $V_{\max}$. If the hydrolysis of the glycosyl enzyme would be rate determining, $V_{\max}$ ought to be the same for both 2-deoxyglucosides (at 25 °C the rate of the p-nitrophenyl derivative would probably be less than half the rate given in Table I). Our failure to detect a burst of phenol does not exclude a two-step mechanism, it merely puts some limitations on the individual rates. The $V_{\max}$ for 4-methylumbelliferyl- β -glucoside corre-

Table I

Hydrolysis of glycosides by β -glucosidase A from sweet almonds at pH 5.0 and 35 °C (p-nitrophenyl derivatives) and 25 °C (4-methylumbelliferyl derivatives), respectively

	K_m (mM)	$V_{\max} \frac{\mu\text{moles}}{\text{min mg}}$
p-nitrophenyl- β -glucoside	2.0	70
- β -xyloside	3.0	0.95
- β -2-deoxyglucoside	4.0	0.1
4-methylumbelliferyl- β -glucoside	1.7	390
- β -xyloside	0.9	3.7
- β -2-deoxyglucoside	0.7	0.17

sponds to a turnover number of 440 sec⁻¹ which should be equal to k_2 . Assuming k_3 to be 50 times larger than k_2 gives 2.2×10^4 sec⁻¹ for the deglycosylation rate constant which is more than two orders of magnitude higher than the deacylation rate of chymotrypsin with specific substrates [13]. The ester mechanism appears, therefore, somewhat questionable. To what extent, now, does the mechanism proposed in Fig. 4 hold for other glycosidases? Conduritol B epoxide reacts with a variety of β -glucosidases [14]. β -Galactosidases are inactivated only when they are active against β -glucosides too, like the hexosidases from snail digestive juice. The β -galactosidase from *Escherichia coli* could be inactivated with an epoxide analogous to galactose [15]. Cellulases did not react but were inactivated by epoxides derived from cellobiose or cellotriase [16]. α -Glucosidase from yeast reacts slowly with conduritol B epoxide but very rapidly with 6-bromo-3,4,5-trihydroxy cyclohexane. A histidine residue is alkylated in this reaction [17]. In addition, two sulfhydryl groups are present at or near the active site.

It is quite likely that with all these enzymes two functional groups are involved in the catalytic step, one acting as an acid to protonate the glycosyl oxygen, the other acting as a base to stabilize a developing carbonium ion. The application of active site directed inhibitors for the study of glycosidases

has only started. Extension of this principle for labelling other groups at the active site will, in combination with carefully planned kinetic experiments, give a clearer picture in the future.

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ORIGINAL PAPERS

NON-RIBOSOMAL BIOSYNTHESIS OF THE CYCLIC OCTADECAPEPTIDE ALAMETHICIN

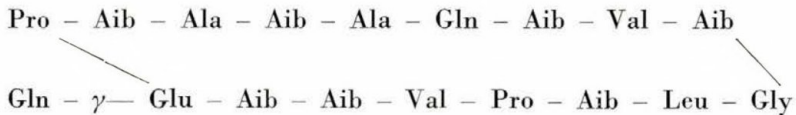
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Summary. The biosynthesis of the cyclic octadecapeptide, alamethicin, in a cell-free system of *Trichoderma viride* has been investigated. It was shown that nucleic acid- and ribosome-free extracts of *Trichoderma viride* could catalyze alamethicin biosynthesis. Puromycin, erythromycin and RNase did not inhibit this synthesis. The Sephadex G 200 filtrate contains a fraction ($K_{av} = 0.1$) that catalyzes the biosynthesis of alamethicin and shows an ATP- 32 PP $_i$ exchange with 6 of the 8 constituent amino acids of alamethicin. The activated amino acids are bound to the enzyme as aminoacyl adenylates and as thioesters in a proportion of 1 : 1. About 50% of each bound amino acid could be split off with 7% TCA. The TCA-stable bound amino acid could be split by mercury acetate, hydroxylamine and performic acid. N-ethylmaleimide blocked the binding of 50% of the amino acids to the enzyme, proving that some of the amino acids first bound as aminoacyl adenylates are then transferred into a thioester bond.

Studies of the biosynthesis of peptide antibiotics have shown that their synthesis occurs by a mechanism independent of nucleic acids and ribosomes [1-7]. The mechanism for the biosynthesis of gramicidin S and the tyrocidines is well established; the constituent amino acids are activated by the formation of aminoacyl adenylates followed by transfer of the aminoacyl residues to thiol groups on the same enzyme [8]. In gramicidin S synthetase I, 18 or 19 catalytic functions are postulated [9]. The arrangement of amino acids takes place on a multienzyme complex. The length of peptide that can be synthesized by this mechanism is unknown. The present work on the biosynthesis of alamethicin has been directed to this problem.

Alamethicin is a cyclic octadecapeptide containing as an unusual amino acid aminoisobutyric acid (Aib) in several positions and has the sequence [10]:



It was first isolated from cultures of *Trichoderma viride* in 1966 [11]. REUSSER [12] showed that cycloheximide failed to inhibit alamethicin biosynthesis *in vivo*, and concluded that ribosomes are unnecessary for this process. In the present work the biosynthesis of alamethicin was studied *in vitro* in the presence of puromycin and RNase. The results clearly indicate that the

synthesis is independent of nucleic acids and ribosomes. The amino acids are activated to form the corresponding adenylates, and subsequently the aminoacyl moieties are transferred to sulfhydryl groups, yielding thioesters on the same enzyme in an intermediate stage of alamethicin biosynthesis.

Materials and methods

Culture of *Trichoderma viride*. The alamethicin producing strain of *T. viride* (a gift of the Upjohn Company, Michigan, USA) was kept in soil cultures, then cultured in flasks by shaking under aerobic conditions at 27°C. The medium had a pH of 6.8; it contained soy bean meal (1.5%), soy peptone (1.5%), dextrin (3%), and D-glucose (2%).

The alamethicin in the culture medium was precipitated by adjusting the pH to 4.6. After centrifugation it was taken up in ethanol and purified by thin-layer chromatography on silica gel in 1-butanol : water : acetic acid (2 : 1 : 1 v/v). The substance co-migrated with authentic alamethicin on electrophoresis. After hydrolysis at 110°C in 6 N HCl for 24 hr, amino acid analysis agreed with the proposed structure.

Preparation of cell-free extracts. The mycelia were harvested at the time of maximum alamethicin production, at the end of the log phase, 60 hr. The buffer (A) used was 0.04 M Tris-HCl, pH 7.4, with 10% sucrose, 0.01 M MgCl₂, and 0.025 M ethylenediamine tetraacetate (EDTA) and 1 mM dithiothreitol (DTT). Twenty g of moist cells were taken up in two volumes of buffer A and forced through a cooled French Press (1500 psi). The paste produced was diluted and centrifuged at 1000 g for 10 min and 100 000 g for 90 min to remove ribosomes. The supernatant was precipitated with 60% ammonium sulphate and the precipitate was resuspended in buffer A and dialyzed against this buffer twice for 2 hr each. The crude extract is stable at -90°C.

Alamethicin biosynthesis in vitro. Enzyme solution, 0.5 ml, was incubated at 37°C for 45 min in 1 ml of a reaction mixture (Mix 1) of 0.1 M triethanolamine hydrochloride (TRA/HCl) (pH 7.8), 1 mM DTT, 5 mM magnesium acetate, 10 mM ATP, 1 mM of the 8 constituent amino acids of alamethicin, except the ¹⁴C-labelled amino acid of which 1 µCi was included. Separation and proof of formation of alamethicin was carried out as described by REUSSER [12].

Gel chromatography, ATP-³²PP_i exchange. Crude extract, 5 ml, was passed through a Sephadex G 200 column (2.6 × 90 cm), equilibrated with buffer A and eluted with the same buffer. Fractions of 4 ml were collected and ATP-³²PP_i exchanges dependent on the constituent amino acids of alamethicin were tested as described by ROSKOSKI *et al.* [13]. The incubation mixture contained 100 µl Sephadex G 200 eluate of the peak fraction and 100 µl of a solution of 0.1 M TRA/HCl (pH 7.8), 1 mM DTT, 2 mM ATP, 2 mM ³²PP_i (about 0.1 µCi), 10 mM Mg²⁺ and 2 mM of one of the alamethicin constituent amino acids.

The mixture was incubated for 15 min at 37°C and the reaction stopped by the addition of 0.5 ml of a solution of 1% acid washed Norit A in 0.1 M Na₄P₂O₇ and 3.5% perchloric acid. After 30 min in the cold the suspension was filtered through a glass fibre filter and washed with 30 ml of 0.1 M Na₄P₂O₇ and 180 ml of water. The filters were dried at 110°C and, after the addition of toluene, radioactivity was counted in a Nuclear Chicago Mark II liquid scintillation counter.

The active fractions were pooled and applied to a DEAE-Sephadex A 25 column (1.5 × 15 cm). After washing with 30 ml of buffer A, the column was eluted with a linear KCl gradient (0-0.25 M). Fractions of 3 ml were collected and ATP-³²PP_i exchanges were checked as described.

Enzyme amino acid substrate. The reaction mixture contained 250 ml of Sephadex G 200 eluate or DEAE Sephadex A 25 eluate, 250 µl of mixture I and 1 µCi of the labelled amino acid. Incubation was carried out at 37°C for 30 min. After incubation, the mixture was separated on Sephadex G 50 (1.5 × 50 cm) and 1 ml fractions were collected. Of every other fraction, 250 µl were tested for radioactivity and the fractions containing the enzyme bound amino acid were pooled. Carrier bovine serum albumin (0.5 ml of a 1% solution) was added and the protein precipitated with trichloroacetic acid (TCA) (final concentration 7%). After 30 min at 0°C, the suspension was centrifuged and the precipitate was washed 3 times with 2 ml of 7% TCA, twice with 2 ml of ether-ethanol (3 : 1), and twice with 2 ml of ether. The precipitate was dried and stored at room temperature.

To test the stability of bonds between amino acids and proteins, the methods of KLEIN-KAUF and GEYERS were used [14]. The precipitates were treated with 0.1 M glycine-NaOH (pH 10, 60°C, 10 min), 0.1 M Na-malecite buffer (pH 6.6) plus 1% mercury acetate (37°C, 10 min), 3 M hydroxylamine (pH 6.1, 37°C, 10 min) 100 μ l each.

The supernatants were lyophilized and the residues extracted with methanol : 1 N HCl (9 : 1). The cleared products were separated by thin-layer chromatography on silica gel plates (solvent, 1-butanol : acetic acid : water 4 : 1 : 1). Radioactivity was detected with a Berthold scanner.

Performic acid oxidation was performed as described in [15]. The liberated amino acid was identified by thin-layer chromatography (see above).

Results and discussion

Total biosynthesis of alamethicin. Ribosome-free extracts of *T. viride* catalyze the biosynthesis of alamethicin. For this the 8 constituent amino acids and ATP were specifically needed (Fig. 1). This suggested the participation of aminoacyl adenylates in the first step of the amino acid activation. RNase, puromycin and erythromycin had no effect on the biosynthesis (Fig. 2). On the contrary, they even seemed to stimulate alamethicin production by inhibiting the ribosomal synthesis of proteins.

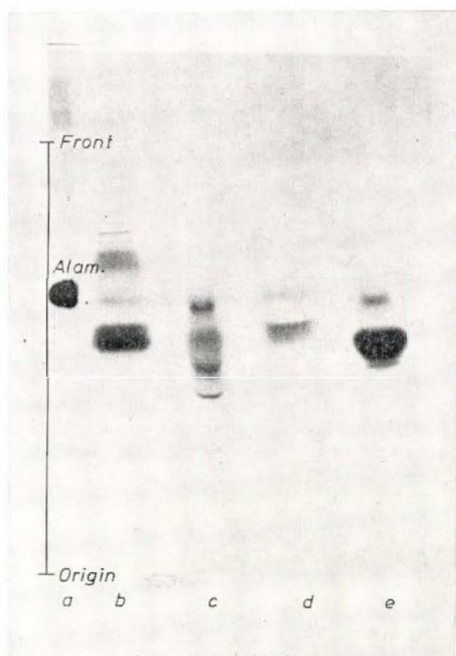


Fig. 1. *In vitro* synthesis of alamethicin. Identification of ^{14}C -labelled product by thin-layer chromatography. ^{14}C -labelled alamethicin (a), incorporation of Aib (b), L-Pro (c), L-Leu (d) and L-Val (e). The incubation conditions were described in the Methods section. The reaction mixture was heated to 100°C for 2 min, then extracted with 2 ml 1-butanol : chloroform (4 : 1). The upper layer was removed and dried *in vacuo*. The residue was taken up in 250 μ l of ethanol and 200 μ l amounts were put on silica gel plates for chromatography, using 1-butanol : acetic acid : water (2 : 1 : 1) as solvent

Amino acid activation. Gel filtration of the crude extract from Sephadex G 200 yields one peak at $K_{av} = 0.1$ that catalyzes biosynthesis of alamethicin. Activation of the constituent amino acids occurs at the aminoacyl adenylate stage before binding to the synthesizing system. Six of the 8 amino acids contained in alamethicin showed an ATP- $^{32}\text{PP}_i$ exchange reaction (Fig. 3). Activation of Glu and Gly could not be detected. Ion exchange chromatography on DEAE-Sephadex A 25 of the peak fractions from the Sephadex G 200

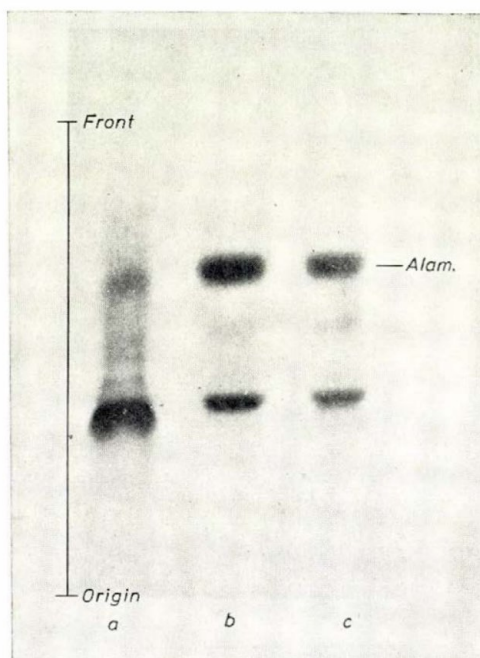


Fig. 2. *In vitro* synthesis of alamethicin and separation by thin-layer chromatography. Incorporation of ^{14}C -Ala: (a) standard incubation; (b) in the presence of 50 μg RNase; (c) in the presence of 50 μg puromycin. The conditions for the extraction of the reaction products were the same as described in the legend for Fig. 1

fractionation yielded one peak at 1.1 mS, which had positive ATP- $^{32}\text{PP}_i$ exchanges and showed alamethicin biosynthesis. There was a second peak at 3 mS, which exhibited an L-Leu-dependent ATP- $^{32}\text{PP}_i$ exchange, but did not stimulate alamethicin biosynthesis (Fig. 4). Comparing the multienzyme complexes [15–18] catalyzing gramicidin S and the tyrocidine biosynthesis, they differ in that the biosynthesis of alamethicin might be catalyzed by one enzyme only. With the methods applied we were unable to fractionate this enzyme into more than one individual, but there might be enzymes with similar physical properties involved in the biosynthesis of this peptide.

Enzyme-amino acid complexes. The mechanism suggested for the biosynthesis of gramicidin S and tyrocidine includes in the first two steps the formation of enzyme bound aminoacyl adenylates, followed by a transfer of the amino acid to a thiol group on the enzyme surface by detachment of AMP. At the site of the enzyme where the adenylate was attached a new molecule of an amino acid is able to react with ATP [14].

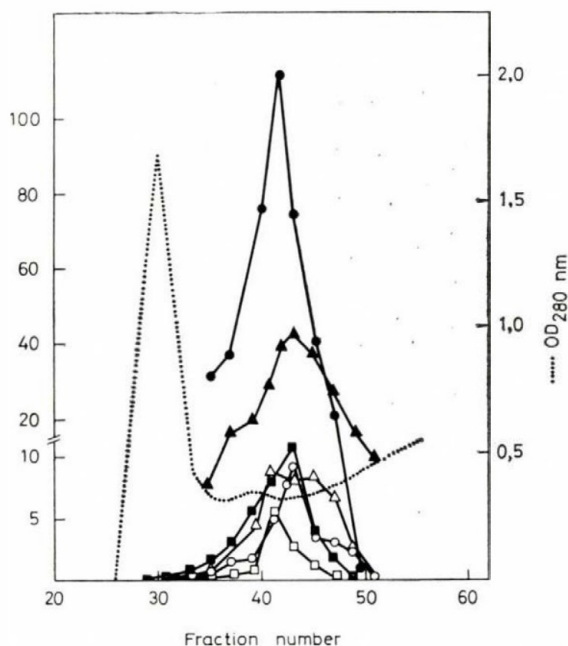


Fig. 3. Separation of the alamethicin-synthesizing system by gel chromatography on Sephadex G 200. Crude extracts, 5 ml, from 10 g wet cells were put on a column (2.6 × 90 cm; $V_0 = 135$ ml) equilibrated with buffer A, and eluted with the same buffer. Fractions of 4 ml were collected, optical density at 280 nm was measured. ATP- 32 PP $_i$ exchanges dependent on L-Leu (●), L-Val (▲), Aib (■), L-Pro (○), L-Ala (△), and L-Glu (□) were determined as described under Methods

This results in a ratio of 1 : 1 between covalently bound amino acids to non-covalently bound amino acids, on the enzyme. The non-covalently bound amino acids can be separated from the enzyme by 7% trichloroacetic acid.

The thioester bonds between enzyme and amino acid showed the following characteristics [14]: they were stable against acids and labile against alkali as well as mercuric salts at neutral pH; hydroxamates were formed in the presence of salt-free hydroxylamine at pH 6.1; borohydride reduction resulted in the formation of amino alcohols; performic acid oxidized the bonds.

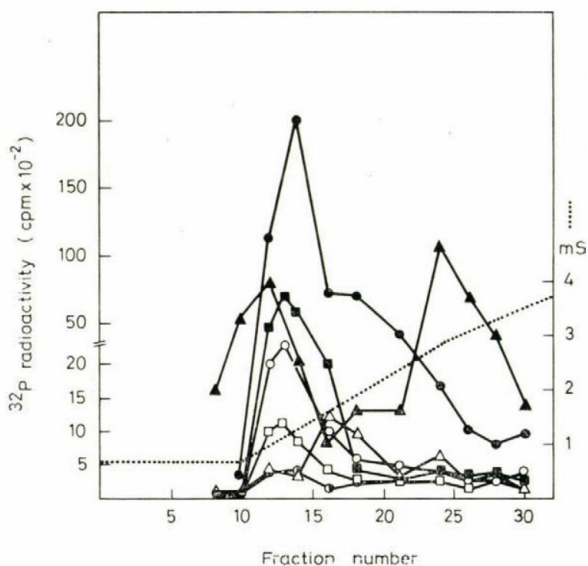


Fig. 4. Separation of the alamethicin-synthesizing system on DEAE-Sephadex A 25. The Sephadex G 200 fractions 35–50 (see Fig. 3) were pooled and applied to a DEAE-Sephadex A 25 column (1.5 × 15 cm), equilibrated with buffer A. After washing with the same buffer (30 ml), the column was eluted with a linear KCl-gradient (0–0.25 M). Fractions of 3 ml were collected, ATP- ^{32}P _i exchange activities of L-Val (●), L-Leu (▲), L-Pro (■), Aib (○), L-Ala (△), L-Glu (□) and amino acid-independent (○) exchange activity were determined as described under Methods

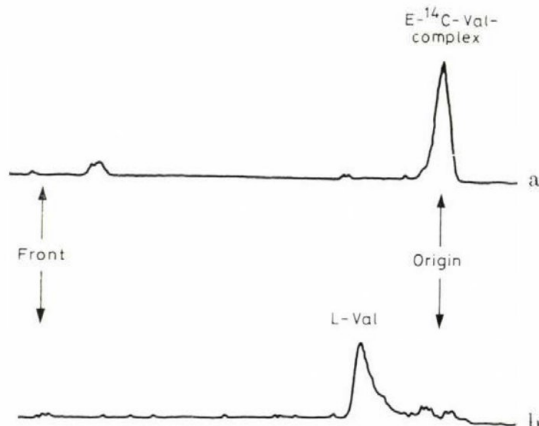


Fig. 5. Liberation of protein-bound radioactivity with performic acid. Untreated complex (a), separation of ^{14}C -L-Val by thin-layer chromatography (b). Solvent, 1-butanol : acetic acid : water (4 : 1 : 1). Radioactivity was detected with a Berthold scanner

Reagents that react with SH-groups inhibited ester formation indicating that the activation had stopped at the aminoacyl adenylate step.

The alamethicin synthesizing system is able to form TCA-stable associa-

tions that correspond to the characteristics mentioned above. For example, in Fig. 5 the cleavage of an enzyme —(^{14}C)-L-Val-complex by performic acid oxidation is demonstrated. The same complex is stable against concentrated formic acid. Fig. 6 shows the influence of the SH-reagent N-ethyl-maleimide (NEM) on the formation of the enzyme —(^3H)-L-Val association. The amino acid attachment is decreased to about 50%. Obviously the transfer of aminoacyl adenylate is blocked. This is a further indication for the activation mechanism mentioned.

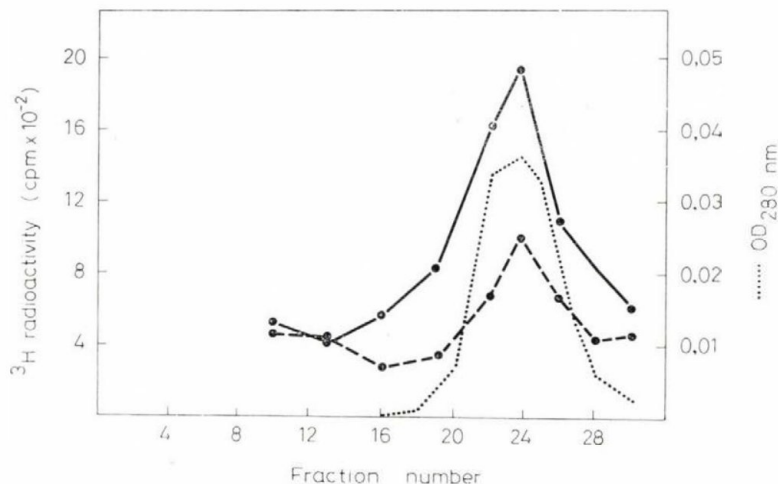


Fig. 6. Binding of ^3H -L-Val to the enzyme in the absence (●—●) and in the presence (●—●—●) of NEM (0.5 mg/ml). The incubation mixture contained 250 μl enzyme solution (Sephadex G 200 eluate: fraction number 43, see Fig. 3), 250 μl Mix 1, 1 μCi ^3H -L-Val and 50 μg RNase. Incubation was carried out at 37°C for 30 min. The mixture was then applied to a Sephadex G 50 column (1.5 $\times$ 50 cm) and eluted with buffer A. Fractions of 1 ml were collected (up to fraction 10 : 3 ml), 10 ml of Bray's solution was added and radioactivity was determined in a Nuclear Chicago Mark II liquid scintillation counter

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SUBSTRATE SPECIFICITY OF THE AMINO ACYL ADENYLATE ACTIVATION SITES OF GRAMICIDIN S-SYNTHETASE (GSS)

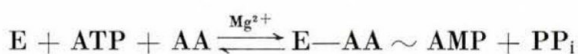
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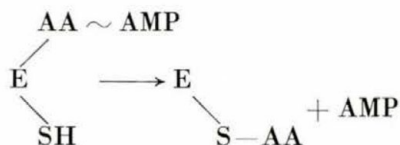
Summary. An investigation was made of the intermolecular forces which determine substrate recognition and binding as well as of the topography and localized environment of the different binding sites of the substrate amino acids of gramicidin S-synthetase (GSS) using substrate derivatives as molecular probes. It is demonstrated that among the aminoacyl adenylate binding sites of the heavy component of GSS the activation site of L-ornithine is distinguished by a relatively high substrate variability. The active centres of GSS are less restrictive for the activation of substrate analogues modified at the carboxyl group than for derivatives substituted at the α -amino group.

The biosynthesis of gramicidin S requires two complementary enzyme fractions (gramicidin S-synthetase, GSS): a light fraction of 100 000 daltons that activates and racemizes phenylalanine, and a heavy fraction of 280 000 daltons that activates the other four amino acids, L-proline, L-valine, L-ornithine and L-leucine [1–3].

The primary activation of these substrates is similar to the amino acid activation by transfer ribonucleic acid (tRNA) synthetases, both leading to the formation of aminoacyl adenylates. This process is accompanied by a reversible ATP-PP_i exchange:



In a second activation step the amino acid substrates (AA) of GSS are translocated to an active SH group at the reaction centres of this multienzyme forming thioesters:



From this stage the amino acids (AA) are linked together by the participation of a phosphopantethein arm (elongation reaction). The last step of the biosynthesis of gramicidin S is the cyclization of two identical pentapeptides finally formed in the elongation reaction (termination and cyclization).

An understanding of the complex mechanism of the biosynthesis of gramicidin S requires the knowledge (a) of the intermolecular forces which determine the substrate recognition and binding, as well as (b) of the topography and localized environment of the different substrate binding sites of GSS.

Such information can, for example, be obtained by a systematical modification of a substrate using the resulting substrate derivatives as molecular probes for the active centre of an enzyme. Studies of this kind concerning GSS have been reported by several authors [4-6].

Testing amino acid substrates with different side chains they found characteristic differences regarding the stereochemical limitations for the binding of these substrate analogues to the aminoacyl adenylate activation sites and the polarity around these reaction centres. We recently started a series of investigations to map the active sites of GSS. The present work is restricted to an analysis of the aminoacyl adenylate activation sites. We mainly examined the effect on the ATP-PP_i exchange reaction of substrate derivatives modified at their amino groups or their carboxyl group.

Materials and methods

Growth of *Bacillus brevis* cells and preparation of the two complementary fractions of GSS were essentially performed as described by GEVERS *et al.* [7] and KLEINKAUF *et al.* [8]. For both enzymes a new additional purification step was introduced after DEAE-chromatography [9]. The heavy component was further purified by density gradient centrifugation using linear 10-30% glycerol gradients.

The light enzyme was finally chromatographed on Aminoethyl-Sephadex column.

The ATP-PP_i exchange reaction was carried out as described in references [8, 10]. The standard assay mixture contained 12.5 μ M triethanolamine-HCl; 0.25 μ M ATP; 0.25 μ M substrate amino acid; 1.25 μ M MgCl₂; 0.0625 μ M EDTA and 0.25 μ M Na₄P₂O₇, in 0.2 ml solution. All assay mixtures were incubated at 37°C. The protein concentration was determined spectrophotometrically by the method of LOWRY *et al.* [11].

The amino acid substrates of GSS were purchased from Merck-Schuchardt GmbH. The N-acetylated compounds and N- δ -t-BOC-L-ornithine were products of Cyclo Chemical. The carbobenzoxy-substituted amino acids and the esters of leucine tested were obtained from Bachem AG. The esters of phenylalanine were purchased from Fluka AG. N- δ -TFA-L-ornithine was prepared by the method of SCHALLENBERG and CALVIN [12].

Results

1. *Substrate specificity of the aminoacyl adenylate activation site of L-ornithine.* In Table I it is shown that L-lysine and some ornithine derivatives substituted at the δ -amino group of L-ornithine are accepted as substrates by the heavy component of GSS. Substitution of the δ -amino group of L-ornithine by the bulky carbobenzoxy residue, however, leads to a completely inactive compound (Table II). The binding constants for L-ornithine and its analogues were determined from double-reciprocal plots by the method of LINEWEAVER

and BURK [13]. In the right column of Table I the negative free energy of binding ($-\Delta G$) for each substance was evaluated from the corresponding K_m -value.

Table I

Activity of L-ornithine and some α -ornithine analogues in promoting ATP-PP_i exchange of the heavy enzyme of GSS

Substrate	K_m (mM)	$-\Delta G$ (kcal/mole)
L-ornithine	0.041	6.20
L-lysine	33	2.09
N- α -Ac-L-ornithine	5.0	3.25
N- δ -t-butyloxycarbonyl-L-ornithine	1.3	4.02
N- δ -trifluoroacetyl-L-ornithine	10.0	2.83
L-arginine	10.4	2.80

The reaction conditions are described in Materials and methods. The K_m -values were derived from Lineweaver-Burk plots.

Table II

Activation of some N-substituted substrate modifications by GSS

Substrate modification	Relative ATP-PP exchange, per cent
N- α -Ac-L-Phe	0
N- α -Ac-L-Pro	0
N- α -Ac-L-Val	0
N- α -Ac-L-Orn	40
N- α -Ac-L-Leu	0
N- α -carbobenzoxyl-L-Orn	0
N- δ -carbobenzoxyl-L-Orn	0

The ATP-PP_i exchange for a tested substrate modification is always referred to the exchange measured for the amino acid from which it was derived. The reaction conditions are described in Materials and methods. Enzyme concentration: approx. 3×10^{-7} M. Concentration of the compounds tested: 2×10^{-3} M.

2. *Substrate and inhibitor studies with derivatives of the substrate amino acids of GSS substituted at their α -amino group.* In Table II the N-acetylated derivatives of L-phenylalanine, L-proline, L-valine, L-ornithine and L-leucine were tested as substrate analogues of GSS. None of the compounds with the exception of N- α -Ac-L-ornithine mediated an ATP-PP_i exchange reaction even at high concentration. In the presence of N- α -Ac-L-ornithine about 40%

of the L-ornithine induced exchange was observed under the conditions described in Materials and methods. Substitution of the α -amino group of L-ornithine by the bulky carbobenzyoxy group also abolished any exchange activity.

From Fig. 1 it is apparent that N-Ac-L-proline is an inhibitor of the proline dependent ATP-PP_i exchange catalyzed by the heavy enzyme. Simi-

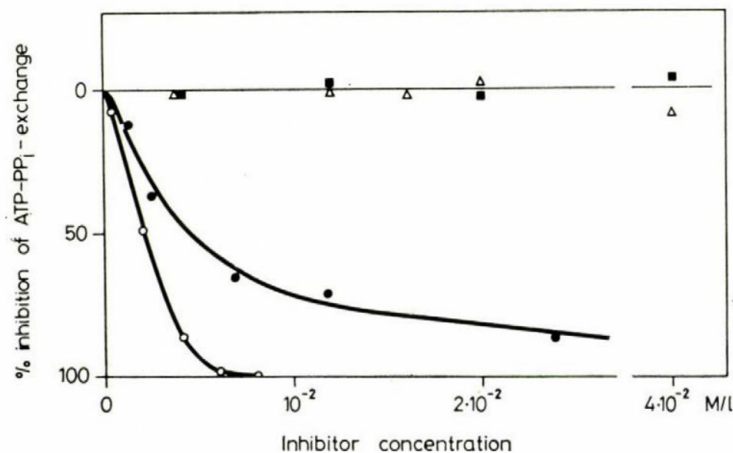


Fig. 1. Effect of N-Ac-L-Phe (●) on the D-phenylalanine dependent ATP-PP_i exchange (light enzyme of GSS) and effect of N-Ac-L-Pro (○); N-Ac-L-Val (■) and N-Ac-L-Leu (△) on the ATP-PP_i exchange mediated by the corresponding substrate amino acid (heavy enzyme of GSS). The reaction conditions are described in Materials and methods. Enzyme concentration: approx. 2.5×10^{-6} (light enzyme) and approx. 2×10^{-7} M (heavy enzyme)

Table III

Activation of esters of L-phenylalanine and L-leucine by the light resp. heavy component of GSS

Amino acid replaced	Substrate modification	Relative ATP-PP _i exchange, per cent
(a) D-phenylalanine		100
	L-Phe-methyl ester	232
	L-Phe-ethyl ester	128
	L-Phe-t-butyl ester	81
	L-Phe-benzyl ester	97
(b) L-leucine		
	L-Leu-methyl ester	22.5
	L-Leu-t-butyl ester	6.5

The reaction conditions are described in Materials and methods. (a) Concentration of the light enzyme: approx. 4×10^{-6} M. Concentration of L-Phe and L-Phe-esters: 0.84×10^{-3} M. (b) Concentration of the heavy enzyme: approx. 5×10^{-6} M. Concentration of L-Leu and L-Leu-esters: 2×10^{-3} M.

larly N-Ac-L-phenylalanine inhibits the phenylalanine dependent ATP-PP_i exchange mediated by the light component of GSS. In contrast N-Ac-L-valine and N-Ac-L-leucine do not influence the valine resp. leucine dependent ATP-PP_i exchange up to the highest obtainable concentrations of these compounds.

3. *Esters of the substrate amino acids as substrate analogues of GSS.* Some esters of L-phenylalanine and L-leucine were tested as substrates of GSS. In Table III it is shown that in the case of the light enzyme the methyl and ethyl esters of L-phenylalanine stimulate the ATP-PP_i exchange reaction as compared to the rate observed for D-phenylalanine, while the ester of higher alcohols, like the t-butyl or benzyl esters, are activated less efficiently. Similar results were obtained for the leucine activation site of the heavy component of GSS. For example, the methyl and t-butyl ester of L-leucine are accepted as substrates by the enzyme.

Discussion

Though the present work needs to be extended by further binding studies and kinetic measurements, it reveals interesting differences in the binding specificity and the stereochemistry of the aminoacyl adenylate activation sites of GSS. Because the first activation step of the amino acid substrates is analogous for tRNA synthetases in ribosomal protein synthesis and for the multienzyme complexes that catalyze the biosynthesis of peptide antibiotics like gramicidin, tyrocidine or edeine, it is of special importance, if similar activation characteristics are observed for the substrates of both classes of enzymes. Some tRNA synthetases have been intensively studied in this respect [14-20]. These investigations yielded valuable information about the design of the active sites of tRNA synthetases and the molecular forces that determine the recognition and binding of their amino acid substrates.

The results concerning the ATP-PP_i exchange measurements with GSS presented in this paper show that among the aminoacyl adenylate activation sites of the heavy enzyme that of L-ornithine is distinguished by a relatively high substrate variability (Table I). If the side chain of L-ornithine is elongated by a CH₂-group as in the case of L-lysine, the *K_m*-value for this analogue is increased about 1000-fold referred to that of L-ornithine corresponding to a loss of binding energy of about 4 kcal/mole. From this result it seems highly probable that there is a specific polar interaction or a hydrogen bond between the δ -amino group of L-ornithine and a counter group at the active site of the enzyme. Obviously the elongation of the side chain of L-ornithine by a methylene group appreciably disturbs this interaction. Derivatives of L-ornithine with relatively large substituents at the δ -amino group can still be efficiently activated by the enzyme. It is especially interesting that the *K_m*-value of N- δ -t-butyloxycarbonyl-L-ornithine with hydrophobic substituents at the δ -amino

group is about 10 times lower than the K_m -values of L-arginine and N- δ -TFA-L-ornithine that bear polar substituents at this point of the molecule.

From these results we infer that the aminoacyl adenylate binding site of L-ornithine is not rigidly closed and that there is a hydrophobic region which extends from the site normally occupied by the δ -amino group of L-ornithine.

In Table II it is further shown that among the α -N-acetylated derivatives of the amino acid substrates of GSS, N- α -Ac-L-ornithine is the only substrate analogue activated by the enzyme. At the ornithine activation site there are obviously smaller stereochemical restrictions near the α -amino group of the bound substrate than at the activation sites of the other substrate amino acids. Inhibitor studies with the α -N-acetylated substrate analogues revealed further differences in the binding specificity of the aminoacyl adenylate activation sites of phenylalanine, L-proline, L-valine and L-leucine.

N- α -Ac-L-phenylalanine and N- α -Ac-L-proline were found to bind to the enzyme as evidenced by their ability to inhibit the activation of D-phenylalanine resp. L-proline in contrast to N- α -Ac-L-valine and N- α -Ac-L-leucine that are inert substances until maximum solubility.

LIPMANN *et al.* [2] showed that L-valine and L-leucine can be replaced as substrates for GSS by other hydrophobic amino acids of similar size. Polar substitution at the side chain of these amino acids results, however, in completely inactive compounds. We therefore conclude that the aminoacyl adenylate binding sites of L-valine and L-leucine can be described as hydrophobic pockets with rigid steric requirements that also exist in the region of the α -amino group of the substrate bound to the activation centre of the enzyme.

In Table III it is demonstrated that, for example, esters of L-phenylalanine and L-leucine with alcohol residues of appreciable size still permit efficient activation by GSS, the aminoacyl adenylate binding site of L-leucine being more restrictive than that of phenylalanine. To detect special interactions of the different alcohol residues with their environment at the active centres of GSS, the K_m -value for these substances must be determined.

tRNA synthetases also bind esters of their amino acid substrates. The results so far known suggest that at the aminoacyl adenylate activation sites of both tRNA synthetases and GSS there are smaller limitations for the binding of substrate analogues modified at the carboxyl group than for derivatives substituted at the α -amino group. For tRNA synthetases it has been shown that the side chain and the α -amino group are important for the binding of the substrate amino acids to the enzyme, while the carboxyl group is not bound. It seems highly probable that similar relations are also valid for the binding to GSS of phenylalanine, L-proline, L-valine, L-ornithine and L-leucine.

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BIOSYNTHESIS OF BACITRACIN ON A PROTEIN THIOTEMPLATE

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Summary. The dodecapeptide bacitracin A is the major constituent of a family of anti-bacterial peptides produced by *Bacillus licheniformis*. The non-ribosomal biosynthesis of bacitracin has been studied in cell-free extracts. Bacitracin synthetase has been fractionated on Sephadex G 200 column into two fractions; both fractions were required for bacitracin biosynthesis. On the other hand, on a Sepharose affinity chromatography column, using L-leucine as ligand, three fractions were obtained; all three were required for bacitracin biosynthesis. During bacitracin synthesis, the enzyme components contain a number of thioester bound peptides. The nature of the peptides suggested that the synthesis proceeds towards the C-terminal end of the molecule. It is assumed that by sequential addition of thioester-bound amino acids, bacitracin A could be synthesized on the surface of the enzyme containing phosphopantetheine.

Studies at the Department of Biochemistry in Oslo as well as in other laboratories have demonstrated that peptide antibiotics, *i.e.* gramicidin S and tyrocidine, are synthesized by a non-ribosomal mechanism which we like to describe as a "Protein thiotemplate mechanism of synthesis" [1]. To extend these studies we have recently turned our attention to the biosynthesis of bacitracin. Bacitracins are a group of peptide antibiotics produced by *Bacillus licheniformis* (Fig. 1). Bacitracin A has 8 L- and 4 D-amino acids. The ϵ -amino group in lysine is linked to asparagine in a heptapeptide ring. The N-terminal isoleucine and cysteine form a thiazoline ring. It has been shown that the amino acids are activated as adenylates on an enzyme complex [2—4] before being incorporated into the bacitracin molecule [4].

Materials and methods

Bacitracin synthetase was isolated from *B. licheniformis* (ATCC 10716) and *de novo* synthesis of bacitracin A from the L-isomers of the appropriate amino acids was performed as described [4]. The activation of the amino acids as adenylates was measured by the [32 P]PP_i—ATP exchange reaction [4].

Results and discussion

The partially purified enzyme has been gelfiltered on a Sephadex G 200 column [4] (Fig. 2). Two fractions were obtained. Both fractions were needed for bacitracin synthesis. The first fraction (Peak I) catalyzed the 32 PP_i—ATP

exchange reaction in the presence of the following amino acids: Ile, Cys, Leu, Glu, Phe, His, Asp, and Asn. The second fraction (Peak II) catalyzed the exchange reaction in the presence of the other two amino acids, Lys and Orn.

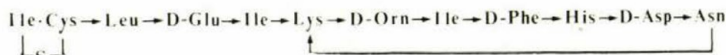


Fig. 1. Bacitracin A

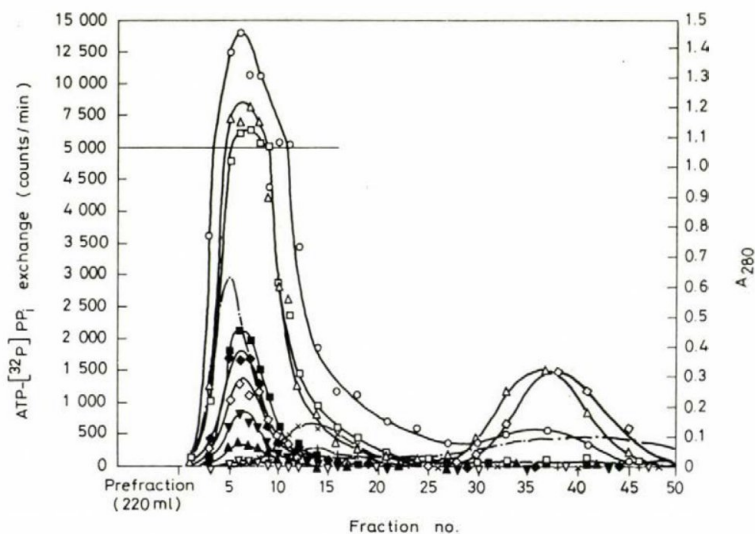


Fig. 2. Fractionation of bacitracin synthetase on Sephadex G 200 column. The 43–49% $(\text{NH}_4)_2\text{SO}_4$ fraction obtained from 10 g wet cells (2 litres culture) was put on a 100×3.2 cm column equilibrated with buffer A [4], reversed flow, at 15 ml/hr. The absorbance at 280 nm ($\bullet$) was measured in each 5 ml fraction. $^{32}\text{PP}_i$ —ATP exchange activity dependent on the L-amino acids isoleucine ($\circ$), cysteine ($\square$), leucine ($\triangle$), glutamic acid (∇), lysine ($+$), ornithine ($\times$), phenylalanine ($\blacktriangledown$), histidine ($\blacksquare$), aspartic acid ($\blacktriangle$), asparagine ($\blacklozenge$) and valine ($\diamond$) was measured in 0.2 ml aliquots of each fraction. Tubes 5–8 and 14–19 were concentrated to 1 ml by ultrafiltration and designated as peak I and II, respectively. The total amount of radioactivity in each incubation mixture was 90 000 c.p.m. The radioactivity (60–80 c.p.m.) measured in control tubes containing no amino acids was subtracted

Aminoacyl-tRNA synthetase could be detected in either of the two peaks. Fig. 3 illustrates that the amino acids were transferred to and bound as thioesters at separate sites on the enzyme surface before being incorporated into the bacitracin molecule. Activation of the first and the last amino acids and not the two ones in the middle makes it probable that the two enzymes are involved in the process.

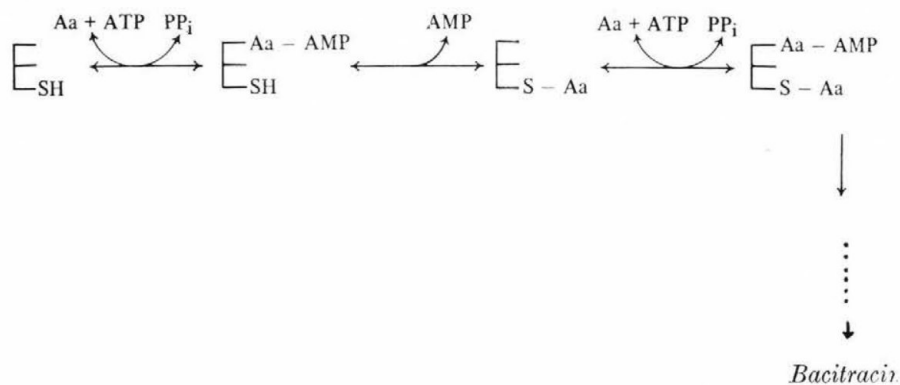


Fig. 3. Activation of amino acids as thioesters

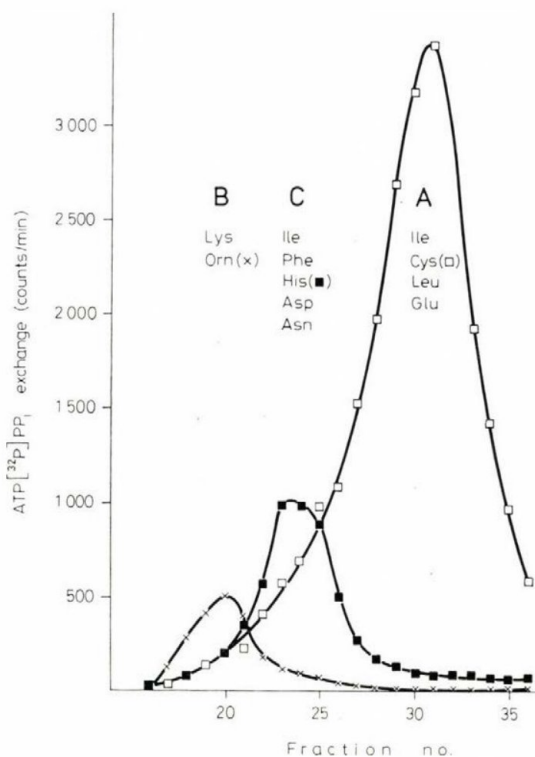


Fig. 4. Fractionation of bacitracin synthetase by affinity chromatography. L-leucine as ligand was bound by its carboxyl group to a Sepharose column by 3,3'-diamino-dipropylamine. The 43-49% fraction was retained on the 12×1.6 cm column and eluted at 40 ml/hr by 0.1-0.4 M KCl solution. $^{32}\text{PP}_i$ -ATP exchange activity was measured in 0.5 ml aliquots of each 10 ml fraction. The total amount of activity in each reaction mixture was 80 000 c.p.m.

By fractionating bacitracin synthetase using affinity chromatography column containing L-leucine as ligand, it was shown that the enzyme consists of at least three components [5] (Fig. 4). All components are necessary for bacitracin biosynthesis. Component A activates Ile, Cys, Leu and Glu, B activates Lys and Orn, while C activates Ile, Phe, His, Asp and Asn. Peak I obtained by Sephadex G 200 chromatography is probably composed of compo-

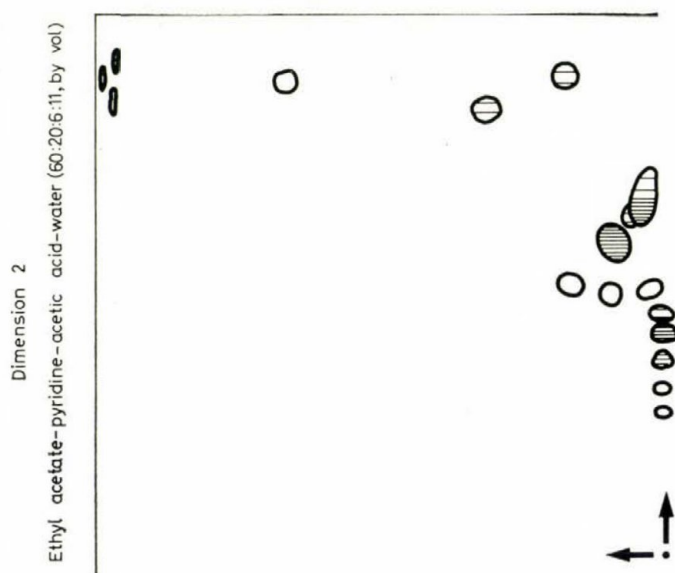


Fig. 5. Two-dimensional chromatogram of ^{14}C -Ile-labelled material liberated from TCA-precipitated protein by performic acid oxidation. Dim. 1, n-butanol : acetic acid : water (4 : 1 : 2, v/v); Dim. 2, ethyl acetate : pyridine : acetic acid : water (60 : 20 : 6 : 11 v/v). The solid support was medium H according to STAHL, E. Merck A. G., Darmstadt, Germany

nents A and C, whereas Peak II is identical with B. It is, however, unclear how the thioester-bound amino acids are incorporated into the bacitracin molecule. To study this, the following experiment was carried out. Bacitracin synthetase was incubated for production of bacitracin in the presence of ^{14}C -labelled isoleucine. The reaction mixture was then gelfiltered on a Sephadex G 50 column. The protein fraction was precipitated with $(\text{NH}_4)_2\text{SO}_4$ and re-incubated for bacitracin synthesis in the presence of ATP, Mg^{++} and all 10 unlabelled amino acids. Table I shows that 96% of the radioactivity remained in the protein fraction after precipitation, out of which 35% was incorporated into bacitracin. Moreover, it appears from Table I that 99% of the radioactivity persisted after TCA precipitation. The TCA stable radioactive compounds could be liberated at alkali pH or by performic acid oxidation and separated by thin-layer chromatography (Fig. 5).

Similar experiments were carried out employing other amino acids in labelled form. Spots containing two up to seven amino acids from the N-terminal end of bacitracin were identified. In addition, spots with remaining amino acids were found. Using the component A which activates the 5 amino acids from the N-terminal end of bacitracin, we expected that the peptide synthesis would stop at the pentapeptide stage. This was exactly what we have found. Experiments are in progress to clarify the interaction between the enzyme components in the peptide synthesis including the ring closure.

Table I

Incorporation of enzyme bound ^{14}C -Ile-labelled material into bacitracin

In the protein fraction after Sephadex G 50 gelfiltration		100%
Experiment	{ $(\text{NH}_4)_2\text{SO}_4$ precipitated Bacitracin formed	96%
No. 1		35%
Experiment	TCA precipitated	99%
No. 2		

It is very likely that the spots represent intermediate peptides in the biosynthesis of bacitracin. The stability towards $(\text{NH}_4)_2\text{SO}_4$ and TCA, and the lability towards alkali pH and performic acid indicate that the intermediate peptides are linked to the enzyme by thioester bound as in the case of gramicidin S [6, 7] and tyrocidine biosynthesis [8].

The resemblance of the mechanisms of synthesis of fatty acids and peptide antibiotics suggests that phosphopantetheine — which is found in both gramicidin S and tyrocidine synthetases [9, 10] — acts by transferring peptide intermediates in thioester linkage to the next thioester bound amino acid on the surface of the enzyme. Pantothenic acid was found in peak I obtained in the course of gelfiltration of bacitracin synthetase complex on Sephadex G 200 [4]. Both the components A and C probably contained the cofactor [11].

Bacitracin seems to be synthesized in the following manner. Amino acids are activated as adenylates on the enzyme surface which consists of at least three components. The activated amino acids then are bound as thioesters at separate sites of the enzyme (protein thiotemplate), where the bacitracin molecule is built up towards the C-terminal end.

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NOTE ADDED IN PROOF

Recent experiments [12] indicate that the peptidation proceeds in the C-terminal direction up to the dodecapeptide stage. The cyclization and release of bacitracin take place probably by the formation of an amide bound between the α -carboxyl group in the thioester bound asparagine and the ε -amino group in lysine.

THE BIOSYNTHESIS OF HYDROXYNALIDIXIC ACID

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Summary. Analytical methods have been worked out for the study of the human metabolism of nalidixic acid. Under pathological conditions the excretion of its metabolites differed from that observed in healthy volunteers. The cause of these variations was studied on the basis of individual excretion curves. The results showed that human and microbial biotransformations were identical. The possibilities offered by the new methods in drug research are discussed.

Shortly after the first description of nalidixic acid (NA) [1, 2], its metabolism was studied by McCHESNEY *et al.* [3] by means of combinations of paper chromatographic and spectrofluorometric methods on healthy volunteers. This was followed by detailed pharmacokinetic studies (Fig. 1). The 7-methyl group of NA is hydroxylated to 7-hydroxymethyl nalidixic acid (HNA) and this is oxidized to 3,7-dicarboxy-nalidixic acid (CNA). The major metab-

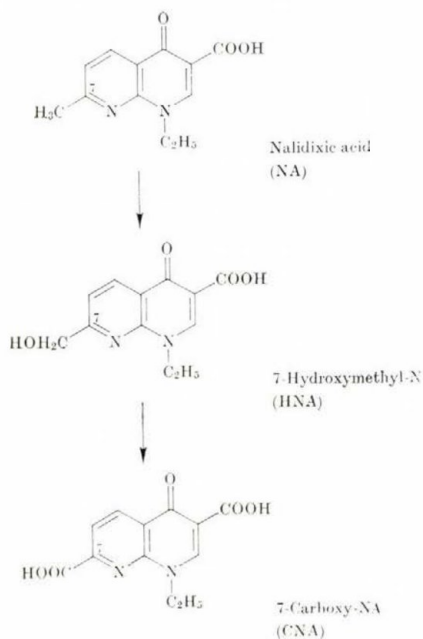


Fig. 1. Human biotransformation of nalidixic acid

olite is HNA; CNA can be regarded as the minor metabolite. These metabolites were isolated and their structure was subjected to analysis and then the compound was synthesized [4–6]. NA and HNA — both closely similar in antimicrobial effect — are excreted to 5–10% with urine, mostly in glucuronized form, as shown by indirect measurements. The antimicrobial effect of CNA is negligible, that of its glucuronide has not yet been reported.

Based on studies performed on healthy volunteers [3, 7–10], the pharmacokinetic behaviour of NA seems to be favourable for the effect of the

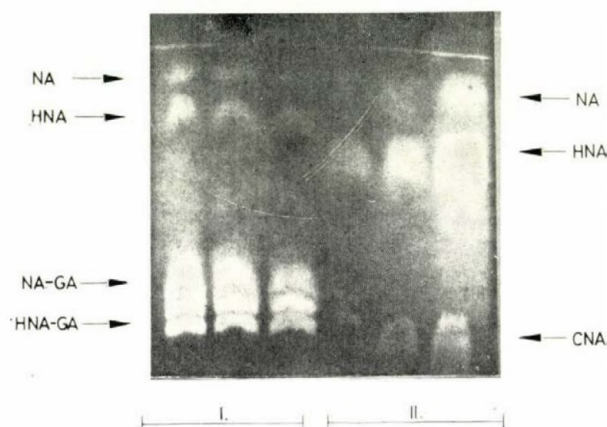


Fig. 2. Paper-chromatographic analysis of nalidixic acid and its metabolites. Solvent: n-PrOH-EtAc-water 6 : 1 : 3 (ascending system). Paper: Schleicher-Schüll 2043 B; I: direct chromatography of urine. II: analysis of chloroform extract from acidified urine (free forms)

basic molecule. In recent years some patents [11, 12] and papers [13–15] have, however, been published concerning the successful transformation of NA → HNA by microorganisms; until that time this conversion could only be achieved chemically with low yields. These studies then revealed that in many cases the human and microbial biotransformations of NA were identical [15]. The importance of these results was further supported by recent reports about structurally different compounds [16–18]. The uniformity of biotransformations, as pointed out by several papers, can be useful in two aspects, *viz.* (i) production of the minor human metabolites by microbial biotransformation, thus avoiding the frequently tiresome isolation procedure; (ii) possibilities of metabolic modelling in microbial systems.

The aim of the present study was to investigate with our own methods the metabolism of NA in healthy persons and in various patients treated occasionally with other drugs, and to check the microbial biotransformation of NA and of other naphthiridine derivatives.

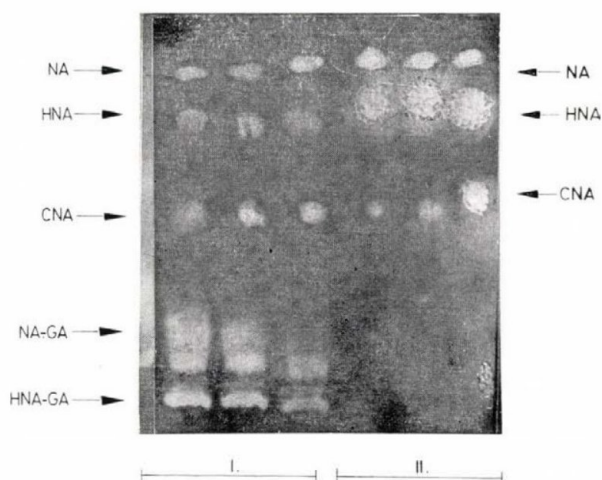


Fig. 3. Paper-chromatographic analysis of nalidixic acid and its metabolites. Solvent: n-butanol-acetic acid-water 4 : 1 : 1 (ascending system). Paper: Macherey-Nagel 214 (for further details see Fig. 2)

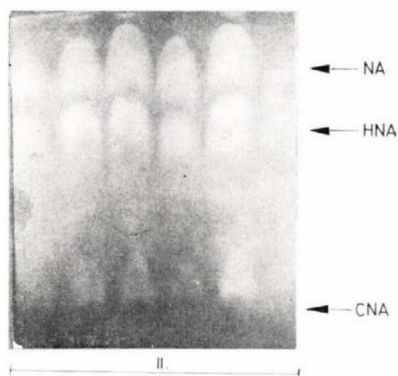


Fig. 4. Paper-chromatographic analysis of nalidixic acid and its metabolites. Solvent: i-PrOH-EtAc-water 4 : 1 : 1. Paper: Schleicher-Schüll 2043 B

Materials and methods

Periodically pooled urine samples were analysed in the course of 24 hr after a single 2.0 g dose, then daily pooled samples were collected and examined after a single 4.0 g dose of the drug.

For separating the basic molecule and its metabolites, 20 different quantitative paper-chromatographic and 5 qualitative thin-layer chromatographic systems have been elaborated. The majority of these systems were suitable for preparative separation with slight modifications. NA and its metabolites were detected under an analytical UV lamp. Some examples of separation can be seen in Figs 2-6, photographed in UV light. NA and its metabolites, HNA, CNA,

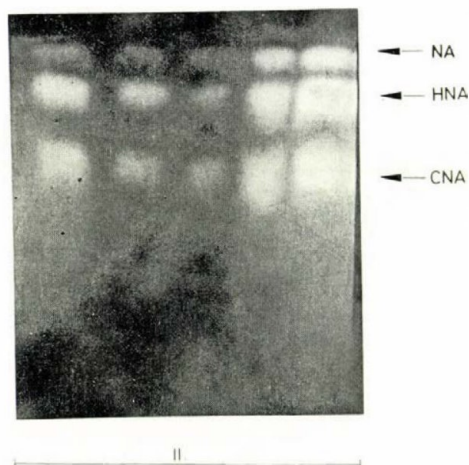


Fig. 5. Paper-chromatographic analysis of nalidixic acid and its metabolites. Solvent: n-butanol-acetic acid-water 4 : 1 : 1 (ascending system). Paper: Macherey-Nagel 214

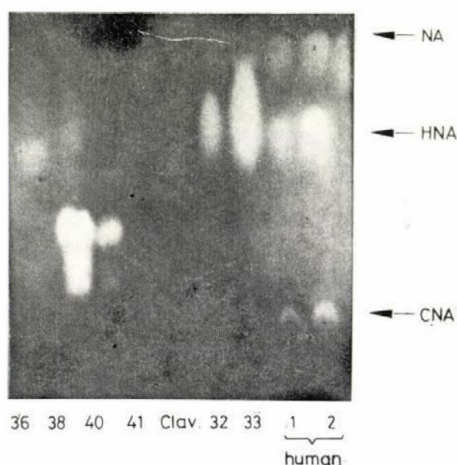


Fig. 6. Paper-chromatographic analysis of nalidixic acid and its biotransformation products extracted from fermentation juices of *Penicillium* and *Aspergillus* strains as well as from human urine. Solvent: i-PrOH-BuAc-EtAc-water 6 : 2 : 1 : 3. Paper: Macherey-Nagel 214

NA-glucuronide, HNA-glucuronide are clearly seen on the figures. Especially important is the proper selection of paper even within one solvent system. *E.g.* for separating the free forms within the n-propanol-ethylacetate-water (6 : 1 : 6) system Macherey-Nagel 214 or Schleicher-Schüll 2043 B papers are more favourable, while for separating glucuronides, Whatman 1 and 3 filter papers are advantageous. It can be seen that on these papers *e.g.* glucuronides give compact spots.

The amount of NA and its metabolites in the spots was measured within the UV-range by absorptiometry (Fig. 7) at 238 or 335 nm, depending on the compounds' concentration in the spot. Free (non-glucuronized) forms in urine were measured following extraction with chloroform after acidification, concentration, and quantitative chromatography.

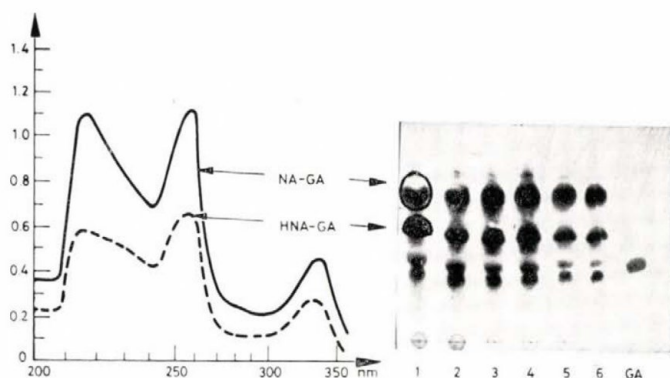


Fig. 7. UV absorption spectra of glucuronides of nalidixic acid and hydroxynalidixic acid. Elution of spots and measurement of spectra were done in 0.1 N NaOH

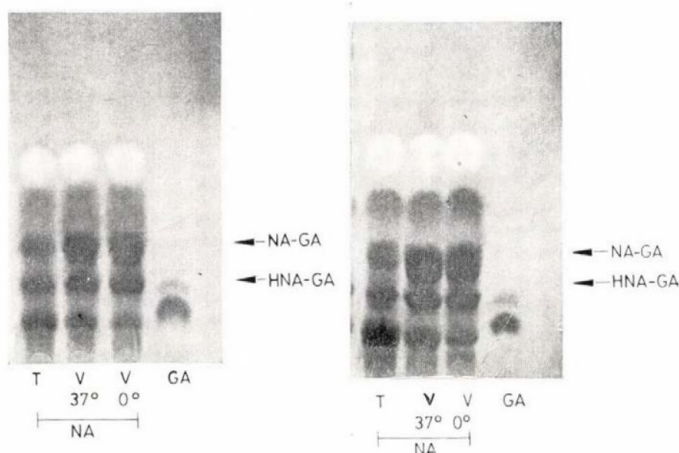


Fig. 8. Paper-chromatographic analysis of nalidixic acid glucuronide (NA-GA), hydroxynalidixic acid glucuronide (HNA-GA) and glucuronic acid. Solvent: n-butanol-acetic acid-water 4 : 1 : 1. Paper: Macherey-Nagel 214. Detection: silver nitrate-sodium hydroxide reagent [19]. $T_{37^\circ} = 2.0$ ml urine + 0.5 ml 0.2 M acetate buffer + 386 I.U. beta-glucuronidase. Incubation: 48 hr at 37°C . $V_{37^\circ} = 2.0$ ml urine + 0.5 ml 0.2 M acetate buffer pH 4.4. Incubation 48 hr at 37°C . $V_{0^\circ} = 2.0$ ml urine + 0.5 ml 0.2 M acetate buffer pH 4.4. Incubation 48 hr at 0°C .
Fig. 9. Paper-chromatographic analysis of nalidixic acid glucuronide (NA-GA), hydroxynalidixic acid glucuronide (HNA-GA) and glucuronic acid (GA). Solvent: n-butanol-acetic acid-water 4 : 1 : 1. Paper: Schleicher-Schüll 2043 B (for further details see Fig. 8)

Identification of substances was based on measurements of m.p., elementary analysis as well as on UV, IR and NMR-spectra.

Glucuronic acid (GA), which is produced upon the effect of NA and excreted in high amounts, as well as NA- and HNA-glucuronides can be visualized with silver nitrate-sodium hydroxide reagent (Figs 8-12). The glucuronic acid content of the NA- and HNA-glucuronides was determined from the water eluates following chromatography [19]. Measurement of the GA content of the eluates was done by colorimetry of the carbazole reaction [20] modified by us.

In order to demonstrate effective antimicrobial agents on chromatograms, bioautographic techniques were applied (Figs 13, 14).

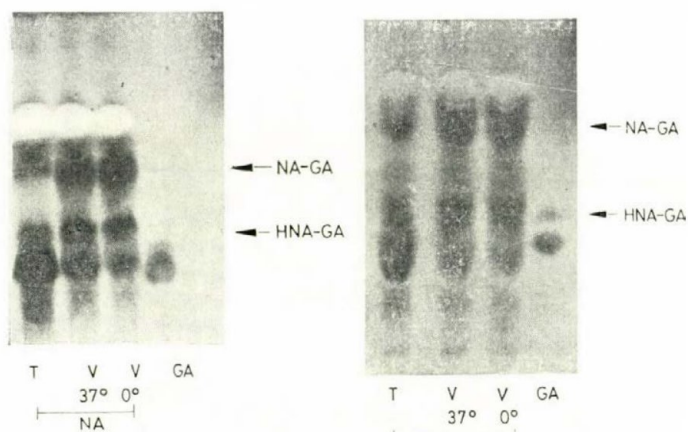


Fig. 10. Paper-chromatographic analysis of nalidixic acid glucuronide (NA-GA), hydroxynalidixic acid glucuronide (HNA-GA) and glucuronic acid (GA). Solvent: *i*-PrOH-BuAc-EtAc-water 6 : 2 : 1 : 3. Paper: Macherey-Nagel 214 (for further details see Fig. 8)

Fig. 11. Paper-chromatographic analysis of nalidixic acid glucuronide (NA-GA), hydroxynalidixic acid glucuronide (HNA-GA) and glucuronic acid (GA). Solvent: *n*-PrOH-EtAc-AcOH-water 5 : 5 : 1 : 3. Paper: Schleicher-Schüll 2043 B (for further details see Fig. 8)

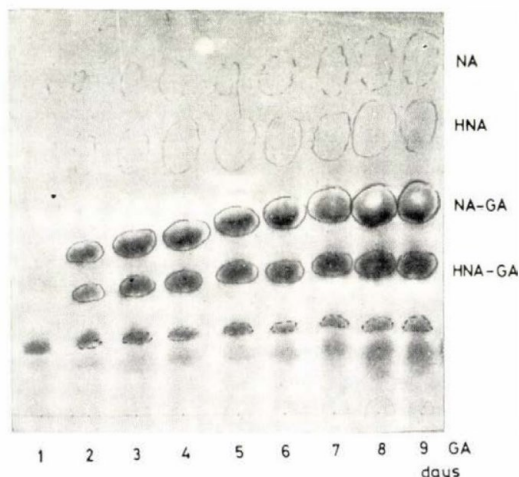


Fig. 12. Paper-chromatographic analysis of nalidixic acid glucuronide (NA-GA), hydroxynalidixic acid glucuronide (HNA-GA) and glucuronic acid (GA). Solvent: *n*-PrOH-EtAc-water 6 : 1 : 3. Paper: Macherey-Nagel 214

Results and discussion

The results have supported those of McCHESNEY *et al.* [3] in that the excretion ratio of NA and HNA was 1 : 3 and 1 : 4, respectively. The ratio of their glucuronized forms was practically identical but with opposite signs.

It can be seen in Fig. 15 that in patient S. K. the excretion of NA-HNA (cumulative curve) was a multiple of the average measured in healthy persons.

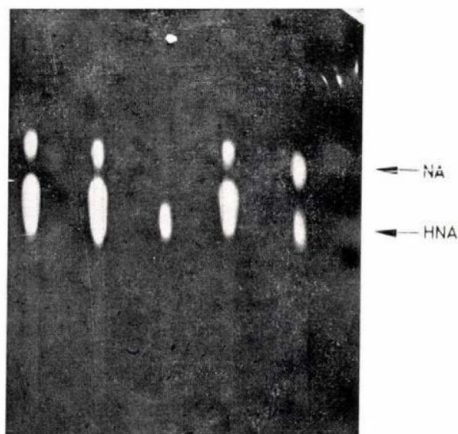


Fig. 13. Bioautographic analysis after paper chromatography. Test organism: *Escherichia coli* 30022 (Hungarian National Collection of Medical Bacteria). Incubation period: 18 hr. Solvent: n-PrOH-EtAc-water 6 : 1 : 3. Paper: Macherey-Nagel 214

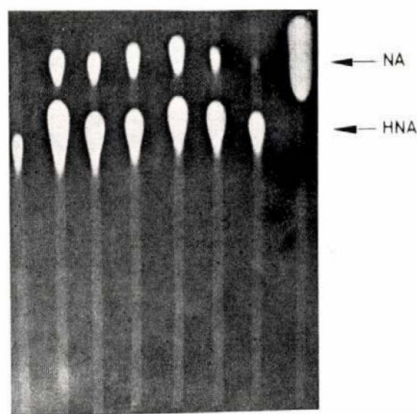


Fig. 14. Bioautographic analysis after paper chromatography (same as in Fig. 13). Solvent: n-PrOH-EtAc-water 4 : 2 : 1. Paper: Schleicher-Schüll 2043 B

It can be seen further that the amount of NA was nearly equal to that of HNA. At the same time the ratio of their glucuronized forms was about the same as that measured in the urine of healthy volunteers.

In patient Sz. L., the proportion and the amount of NA-HNA were nearly normal, while the excretion rate of NA-GA was increased.

In the case of B. V. (Fig. 16) excretion of the total free NA and HNA as well as of their glucuronides were much lower than the previous values, and the ratios for both forms were about 1 : 1.

In T. J., a patient suffering from an inoperable cancer of the head of the pancreas, the glucuronizing capacity was exhausted by the excess bilirubin

and the excretion rate could only be estimated six hours after NA administration.

Table I demonstrates the total amount of NA and its metabolites excreted within 24 hr following the oral dose of NA, as well as the amount of excreted NA-GA and HNA-GA and their ratios. The high individual variations

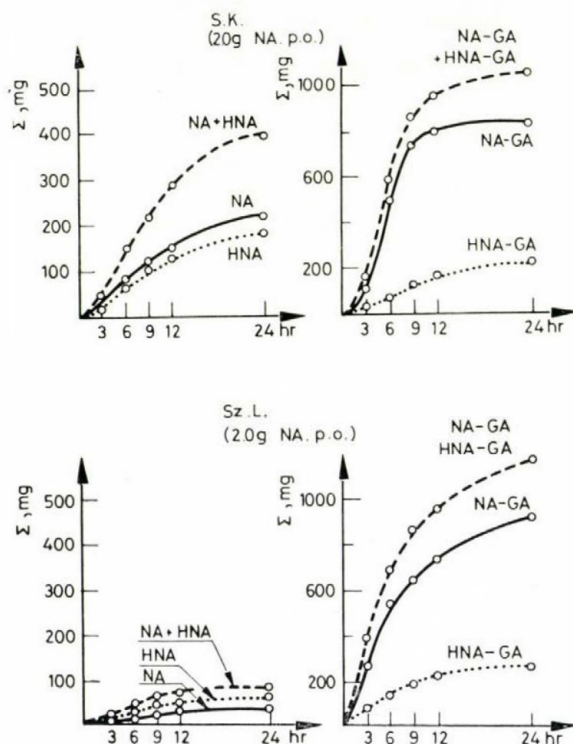


Fig. 15. Concentration of NA and its metabolites in periodically collected urine samples after oral administration of 2.0 g NA (cumulative curves)

in the NA-biotransformation were remarkable and especially so in the ill subjects. As an explanation it might be assumed on the basis of similar biotransformations [21–25] that oxidation of the 7-methyl group, *i.e.* the biosynthesis of HNA, is bound to the “mixed function oxidase” system. Recent data concerning this system [26–29] may explain several aspects of the appearance of HNA *in vivo*. Thus, (i) the biosynthetic variations are determined genetically. (ii) A determining factor may be the accumulation of some endogenous substances (steroid, bilirubin, etc.) [27, 30, 31]. (iii) The function of the system may be influenced by inductor and inhibitor substances [24, 27, 32]; their presence during NA treatment apparently alters the formation of HNA.

(iv) The formation of CNA by dehydrogenase enzymes is in interaction with HNA biosynthesis.

Besides the "mixed function oxidase" system another enzyme system is also of primary importance. It has an influence on glucuronide conjugation and within this on the activity of UDP-glucuronyl-transferase [33-35]. This

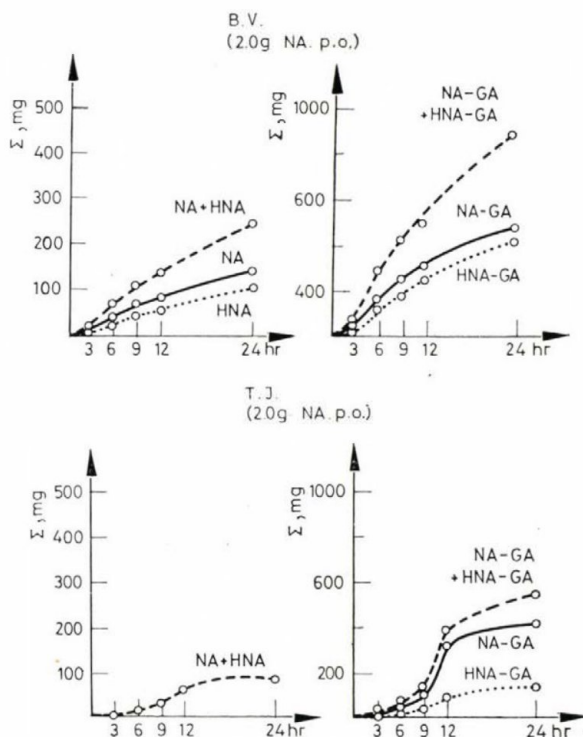


Fig. 16. Concentration of NA and its metabolites in periodically collected urine samples after oral administration of 2.0 g NA (cumulative curves)

system is mainly responsible for the ratio of free HNA and its glucuronide in urine. Its activation, inhibition, substrate supply, etc., may disturb the formation and urinary excretion of HNA even in healthy persons.

Beta-glucuronidase may also be essential in the case of chemotherapeutics excreted in glucuronide form. The beta-glucuronidases of microorganisms living in the organism [36, 37] may split the previously formed HNA-GA complex, and this may explain that in some patients the free level is high and the glucuronide level is low (see the case of patient S. K.).

The data reported in the literature concerning the microbiological transformation of NA have been confirmed by our results, especially the important fact that several microorganisms transform NA in the same way as they are

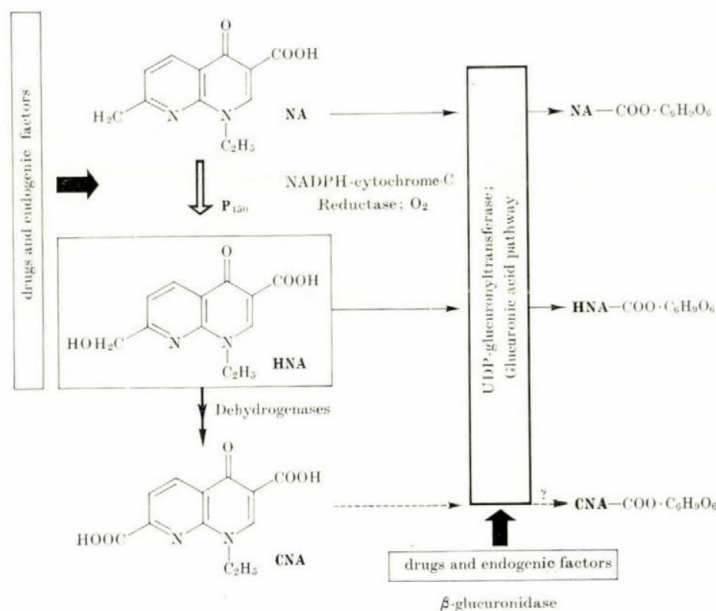


Fig. 17. Human biotransformation pathway of nalidixic acid

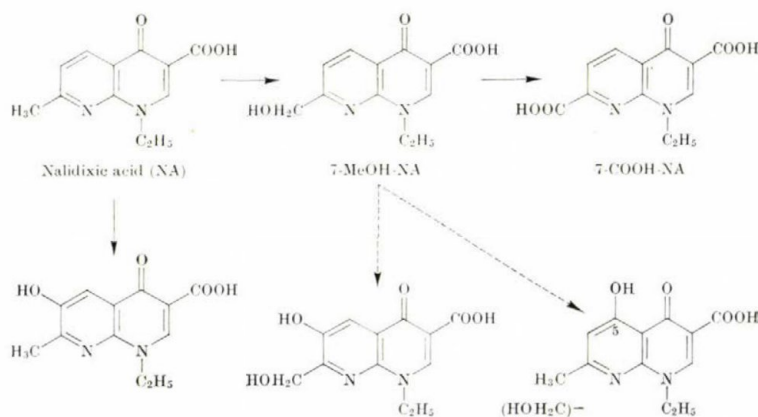


Fig. 18. Microbial biotransformation of nalidixic acid

transformed in the human organism. In Table II some of our strains are shown; the transformation effected by them was influenced by the condition of the strains before inoculation; the conditions of fermentation, pH, temperature, type and concentration of carbohydrate, di- and trivalent cation content among others; NA concentration in the transforming system and the duration of transformation.

It can be seen from Table II that the qualitative NA-transforming ability of these strains may be grouped as follows.

1. Strains performing transformation only to HNA, *e.g.* *A. niger* 34, *P. atramentosum* 201;

2. those completing the synthesis of CNA only, *e.g.* *A. clavatus* 49, *Mucor albo-ater* 7;

3. those producing both HNA and CNA, *e.g.* *A. wentii* 196, *P. decumbens* 216;

4. those producing in addition to HNA and CNA also other, partly unidentified NA-derivatives, *e.g.* *P. citricum* 211.

Table I

Excretion of NA-GA and HNA-GA and their ratio after ingestion of 2.0 g NA

Patient	Σ mg (total) 0-24 hr	Σ mg NA-GA 0-24 hr	Σ mg HNA-GA 0-24 hr	$\frac{\text{NA-GA}}{\text{HNA-GA}}$
B. V.	1099.1	459.9	399.4	1.15
S. B.	1130.1	604.0	472.3	1.27
L. E.	1100.4	663.3	323.7	2.04
H. F.	1378.9	883.0	252.0	2.50
G. H.	1802.6	1259.9	407.4	3.09
T. B.	597.4	408.0	116.1	3.50
Sz. L.	1236.1	899.5	248.0	3.60
S. B.	1444.4	826.5	227.5	3.60
Sz. L.	1846.6	1281.6	328.8	3.81
C. K.	1695.2	1272.6	329.6	3.86
B. L.	2000.6	1631.1	229.3	7.11

In Fig. 18 the data known from the literature and those obtained by us concerning the biotransformation of NA are summarized [13-15]. Beside the two mentioned in the literature, two additional NA-derivatives have been identified. Their assumed structures are shown in Fig. 18.

Recent data in the literature have raised the possibility of modelling mammalian drug metabolism in microbiological systems [16, 38-42]. This would be of importance for studying the NA→HNA reaction. A still higher perspective is also concealed in microbiological biotransformation systems. The metabolism of some inactive derivatives, *e.g.* naphthiridines, or those with poor antimicrobial activity could also be investigated in microbiological transformation systems. These would allow a preliminary study of the correlation of structure and metabolism in microbial models; a study of the biotransformation of toxic derivatives, a question that could not be clarified

Table II
Microbial transformation of nalidixic acid

Species, strain No.	HNA	CNA	NA-derivatives
<i>Aspergillus niger</i> 34 (ATCC 1358)	+		
<i>Aspergillus niger</i> 140		+	+
<i>Aspergillus nidulans</i> 134	+		
<i>Aspergillus nidulans</i> 40	+		+
<i>Aspergillus oryzae</i> 28	+		
<i>Aspergillus clavatus</i> 49		+	
<i>Aspergillus ustus</i> 185			+
<i>Aspergillus wentii</i> 196	+	+	
<i>Aspergillus</i> sp. 175		+	+
<i>Penicillium avellaneum</i> 204	+	+	
<i>Penicillium atramentosum</i> 201	+		
<i>Penicillium aurantio-virens</i> 202	+		
<i>Penicillium citricum</i> 211	+	+	+
<i>Penicillium decumbens</i> 216	+	+	
<i>Penicillium chrysogenum</i> 40	+		+
<i>Penicillium chrysogenum</i> 41 (N)		+	+
<i>Penicillium chrysogenum</i> 42 (W)	+		
<i>Penicillium chrysogenum</i> 43 (A/1)	+	+	+
<i>Penicillium brevicompactum</i> 49	+		+
<i>Claviceps fusiformis</i> 6	+		
<i>Mucor albo-ater</i> 7		+	
<i>Rhizopus oryzae</i> 21	+	+	
<i>Cunninghamella</i> sp. 35		+	+
<i>Rhodotorula rubra</i> 1185		+	+

in animals; investigations of the antimicrobial effect of new derivatives formed in microbiological biotransformation systems would open a new way for studying the relation of structure and optimum effect in drug research.

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METABOLIC FATE OF CHOLESTERYL METHYL ETHER IN MYCOBACTERIUM PHLEI

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Summary. *Mycobacterium phlei* transformed cholesteryl methyl ether into three metabolites: 3 β -methoxy-dinor-5,17(20)-choladien-22-oic acid methyl ester (I), 3 β -methoxy-5-androsten-17-one (II), and 3 β -methoxy-dinor-5-cholen-22-ol (III). After isolation with thin-layer chromatography, their structures were elucidated by mass, IR and NMR spectroscopy. Compound II was the major product. Compounds I and III were products of various side reactions. In the presence of 8-hydroxyquinoline that inhibits degradation of the steroid nucleus, 1,4-androstadiene-3,17-dione was formed in addition to the compounds mentioned. This indicates that a moderate splitting of the ether bond takes place.

In the past few years — parallel with other research groups [1, 2] — we have developed a microbiological process for degradation of the cholesterol side chain, using a strain of *Mycobacterium phlei* [3, 4]. 9 α -Hydroxylation, which would lead to degradation of the steroid nucleus, was prevented by 8-hydroxyquinoline. The product of the transformation was 1,4-androstadiene-3,17-dione. This compound is a suitable material for the manufacture of estrone and 19-norsteroids. In the fermentation broth, in addition to this compound, we have found only 4-androstene-3,17-dione but no intermediates containing a side chain.

Using [^{14}C]cholesterol, 6 β ,19-oxido-4-cholesten-3-one and 19-hydroxy-4-cholesten-3-one as substrates, SIH *et al.* [5, 6] established the degradative sequence of the hydrocarbon side chain in the case of *Nocardia restrictus*. They proved the participation of C-25 and C-22 acid intermediates in the breakdown of cholesterol (C-27).

In this paper we report the results of the transformation of cholesteryl methyl ether by *M. phlei*. The products isolated fit into the pathway established by SIH *et al.* Nevertheless, they indicate some special features of *M. phlei*.

Materials and methods

Fermentation of cholesteryl methyl ether. Cholesteryl methyl ether was synthesized according to SROLL [7]. *Mycobacterium phlei* (MNG No 29) was grown aerobically in 10 litre jar fermenters with 5 litres of neutral growth medium containing 2% cornsteep-liquor (50% dry weight) and 1% glycerol, at 37°C for 40 hr. Then the cells were harvested, resuspended in a 0.4% sodium chloride solution, and 1 g of cholesteryl methyl ether was added to each 5 litres of the cell suspension. Transformation was carried out at 37°C for 3 days.

methoxyl group from the molecular ion affords the fully deoxygenated ion $m/e = 281$. The formation of ions $m/e = 285$ and $m/e = 128$ can be deduced from the rearranged molecular ion I'. These ions supply direct evidence on the structure of the side chain in I.

Compound I showed an UV absorption band at $\lambda_{\max} = 230$ nm, indicating an α,β -unsaturated ester group [8]. IR absorption bands characteristic of conjugated esters were observed at 1710 cm^{-1} ($\nu\text{C}=\text{O}$) and 1620 cm^{-1} ($\nu\text{C}=\text{C}$). In the NMR spectrum of I, the signals were assigned as follows: C-18 and C-19 methyl protons, 0.96s and 1.03s; C-21 methyl protons, 1.96s; $\text{CH}_3\text{—O—}$, 3.37s; $\text{CH}_3\text{—O—CO—}$, 3.71s; $=\text{CH—}$, 5.40 m.

All the above data agree with structure 3 β -methoxy-dinor-5,17(20)-choladien-22-oic acid methyl ester for compound I.

Compound II (R_F 0.55 in system A; m.p. 141°C ; $[\alpha]_D$ 0° ($c = 1.0$, chloroform)) was the major metabolite of cholesteryl methyl ether (yield, 20%). It proved to be identical in all respects with the authentic 3 β -methoxy-5-androsten-17-one prepared according to BUTENANDT and GROSSE [9].

Compound III (R_F 0.45 in system A) was recrystallized from ether giving m.p. $153\text{--}156^\circ\text{C}$. The structure of compound III was deduced from its MS behaviour illustrated by Fig. 2.

Fission A, which involves the rearrangement of an H atom to the fragmentation, and loss of methanol are common processes in every ring-A-saturated steroidal 3-methyl ether [10a]. Fission B is a process characteristic of steroids

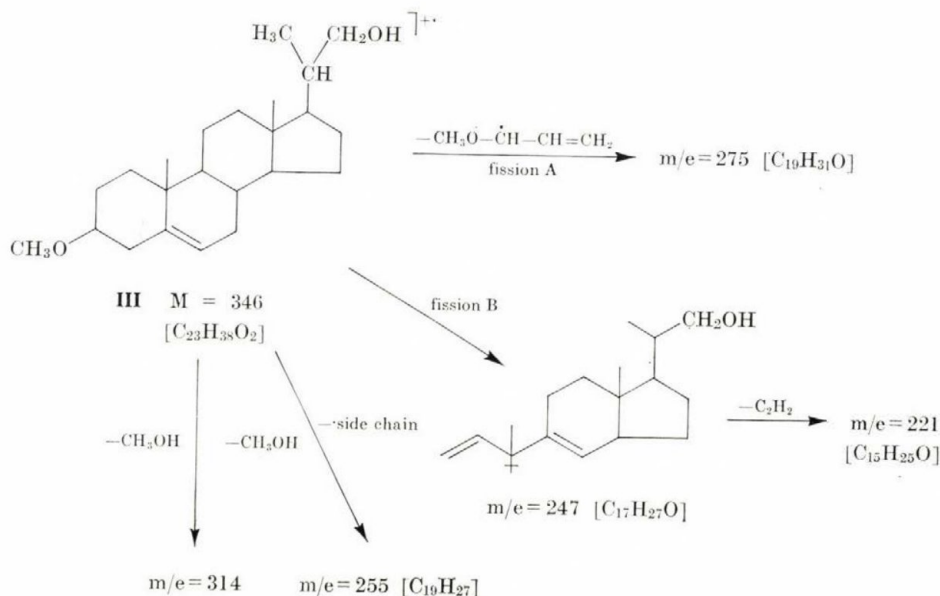


Fig. 2. Mass spectral fragmentation pattern of compound III

containing a 5,6-double bond [10b]. Direct information on the structure of the side chain is furnished by ion $m/e = 255$, which is formed by the loss of methanol and of this moiety.

The IR spectrum of III showed a ν_{OH} band at 3430 cm^{-1} . After acetylation of III with acetic anhydride in pyridine at room temperature, an ester band ($\nu_{C=O}$) appeared at 1730 cm^{-1} in the IR spectrum. In the NMR spectrum of III, the signals were assigned as follows: C-18 and C-19 methyl protons, 0.71s and 1.02s; C-21 methyl protons, 1.06d ($J = 6\text{ Hz}$); C-3 H, sextet ($J_{aa} = 8\text{ Hz}$, $J_{ac} = 4.5\text{ Hz}$); $\text{CH}_3\text{—O—}$ 3.36s; $\text{—CH}_2(\text{OH})$ 3.55m; $=\text{CH—}$ 5.35m.

Consequently, the structure of compound III is 3β -methoxy-dinor-5-cholesten-22-ol.

The degradation pathway of the sterol side chain has been described by S IH *et al.* [5, 6]. They showed that the side chain degraded step by step through

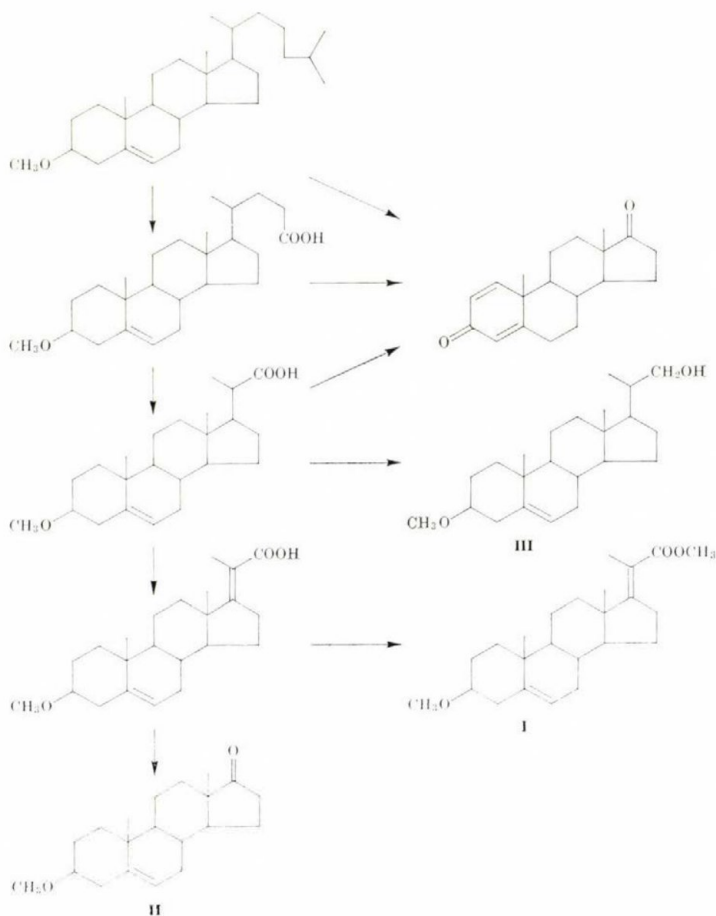


Fig. 3. Degradation of cholesteryl methyl ether

acid intermediates. In accordance with their results, we propose a pathway for the degradation of cholesteryl methyl ether (Fig. 3). We assume that a sterol having C-3 methoxyl group offers a less favoured substrate for the enzymes of *M. phlei*, thus slowing down the breakdown of the side chain. Under these conditions, various side reactions occur. This may be the reason why compound III, a product of reduction of a side chain acid, and compound I, an esterified side chain acid, are formed.

Compound II is the expected product of the degradation, representing indeed the bulk of the extracted products. This compound may be a suitable starting material for a variety of androstane compounds.

In the presence of 8-hydroxyquinoline (100 $\mu\text{g/ml}$), accumulation of 1,4-androstadiene-3,17-dione was also observed in the broth. This means that the ether bond at C-3 is cleft by the microorganism and the demethylated intermediates are degraded via the known pathway [11, 12]. 8-Hydroxyquinoline stops this degradation before 9 α -hydroxylation trapping 1,4-androstadiene-3,17-dione. Cleavage of the ether bond at C-3 must, however, take place at an early stage of the degradation of the side chain, because compound II is reluctant to yield 1,4-androstadiene-3,17-dione even after prolonged incubation with *M. phlei* cells.

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MICROBIOLOGICAL HYDROXYLATION OF NORETHISTERONE

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Summary. Hydroxylation of norethisterone by a large number of fungi has been investigated. 1α -Hydroxy-, 6β -hydroxy-, 10β -hydroxy-, $6\beta,10\beta$ -dihydroxy-, $10\beta,11\beta$ -dihydroxy- 15α - and 15β -hydroxy-derivatives were formed from norethisterone. The microbiological dehydrogenation of 10β -hydroxy-norethisterone resulting in $10\beta,17\beta$ -dihydroxy- 17 -ethynyl- $1,4$ -estradien-3-one was also observed. The structure of transformation products was established by chemical and spectroscopical methods.

In this paper we report on the microbiological transformation of norethisterone (17 -ethynyl- 17β -hydroxy- 4 -estren- 3 -one, I), a commonly used contraceptive. The aim of this work was to prepare hydroxylated derivatives of norethisterone. The hormonal effects of these compounds were studied to determine the alterations in their biological spectra as compared to that of norethisterone.

Materials and methods

The fungal strains tested for hydroxylation of norethisterone during our screening programme were obtained either from the National Institute of Public Health, Budapest or isolated from soil with a method described previously [1]. The isolates were classified by Dr. E. NOVÁK, National Institute of Public Health, Budapest. In the Δ^1 -dehydrogenation experiments *Corynebacterium simplex* ATTC 6946 strain was used.

Fermentation conditions. The microorganisms were maintained on potato glucose agar slants, with the exception of *Sclerotinia sclerotiorum* which was maintained on malt peptone agar slants. Cultivation of the microorganisms was carried out in 500 ml Erlenmeyer flasks containing 100 ml of medium on a rotary shaker at 280 rpm. Four different media were applied.

Medium A contained (per litre of tap water): peptone, 8 g; dextrose, 18 g; malt extract, 8 g; corn steep liquor (dry weight), 2 g; NaCl, 2 g. Medium B contained (per litre of tap water): soybean meal, 10 g; malt extract (dry weight), 30 g; peptone, 10 g; KH_2PO_4 , 5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g; yeast extract (dry weight), 0.2 g. Medium C contained (per litre of tap water): corn steep liquor (dry weight), 9 g; soybean meal, 3 g; dextrose, 7 g. Medium D contained (per litre of tap water): yeast extract (dry weight), 20 g; KH_2PO_4 , 4.4 g; $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 8.8 g.

Absidia orchidis, *Fusarium nivale*, *Mycogone rosea* and *Rhizopus arrhizus* were cultured at 28°C in medium A; *Acremonium kiliense*, *Acremonium strictum*, *Aspergillus fumigatus*, *Cephalosporium asperum* and *Sclerotinia sclerotiorum* at 24°C in medium B; *Cunninghamella elegans* and *Fusarium lateritium* at 24°C in medium C. The cultures of these fungi were employed in the hydroxylation experiments after about 2 days propagation. *Corynebacterium simplex* was cultivated at 37°C in medium D and the resulting culture was incubated with hydrocortisone (10 mg/100 ml culture) for 2 hr in order to induce the Δ^1 -dehydrogenase enzyme, then it was diluted tenfold before being used in Δ^1 -dehydrogenation experiments.

The substrate was added to the cultures in dimethylformamide solution. The amount of substrate added to a given fermentation varied according to the nature of the microorganism between 0.02–0.1 g/100 ml of fermentation medium. The cultures were incubated with the sub-

strate under the same conditions as those used for their cultivation. The progress and completion of each transformation were determined by thin-layer chromatographic analysis of the ethyl acetate extract of samples taken from the bioconversion beer. The analyses were conducted on silicagel chromatoplates using ethyl acetate : n-heptane (7 : 3) as a solvent system. The substrate and the products were detected via ultraviolet light absorption and with concentrated H_2SO_4 .

Isolation of the bioconversion products. At the end of the transformation the fermentation beers were filtered and the filtrates were extracted several times with ethyl acetate. The extracts were evaporated *in vacuo*. In order to separate the conversion products, the residues obtained were chromatographed on preparative silicagel thin-layer plates in ethyl acetate : n-heptane (7 : 3) and in chloroform : ethanol (9 : 1). The isolated products were recrystallized several times before being subjected to structure determination and to biological testing.

Equipment. Melting points (uncorrected) were determined with a Kofler hot stage microscope apparatus. UV spectra (in ethanol) were recorded on a Unicam model SP 500 spectrophotometer. IR spectra (in KBr pellets) with a Perkin Elmer 457 instrument. PMR spectra were determined with Varian A-60D and XL-100-15 spectrometers using tetramethylsilane as an internal standard of reference. Mass spectra were taken on a Varian MAT SM-1 instrument.

Results and discussion

We have attempted the hydroxylation of norethisterone with 312 fungal strains. Only a restricted number of these strains metabolized norethisterone. We selected the 8 most promising strains for further preparative investigation. Conversion of norethisterone by these cultures resulted in the formation of 7 hydroxylated derivatives in significant amounts (4 to 47% yield), as shown in Table I.

Table I

Microbiological preparation of hydroxyl derivatives from norethisterone

Fungi	Hydroxyl derivatives formed from norethisterone						
	1 α	6 β	10 β	6 β ,10 β	10 β ,11 β	15 α	15 β
<i>Acremonium kiliense</i>	+	—	+	—	—	—	—
<i>Acremonium strictum</i>	+	—	—	—	—	—	—
<i>Aspergillus fumigatus</i>	—	—	+	—	—	—	+
<i>Cephalosporium asperum</i>	—	—	+	+	—	—	—
<i>Cunninghamella elegans</i>	—	+	+	+	+	—	+
<i>Fusarium lateritium</i>	—	+	+	—	—	—	—
<i>Fusarium nivale</i>	—	+	—	—	—	+	—
<i>Rhizopus arrhizus</i>	—	—	+	—	—	—	—

The most commonly occurring transformation product (II; mp. 263–265°C; $[\alpha]_D -13.5^\circ$ (c = 1, dioxane)) proved to be identical with synthetic 10 β -hydroxy-norethisterone prepared previously [2, 3]. The best strain for microbiological preparation of II was *R. arrhizus* affording II with 47% yield from I [4]. We found that, in addition to the strains indicated in Table I, several other fungi such as *A. orchidis*, *M. rosea* and *S. sclerotiorum* produce 10 β -

hydroxy-norethisterone from norethisterone [4]. SCHUBERT *et al.* also described the formation of 10 β -hydroxy-norethisterone from 17-ethynyl-1,3,5(10)-estra-1,3,17 β -diol as well as from norethisterone by *Aspergillus flavus* [5].

DE FLINES *et al.* reported the microbiological dehydrogenation of 10 β ,17 β -dihydroxy-4-estren-3-one to 10 β ,17 β -dihydroxy-1,4-estradien-3-one [6]. We observed a similar reaction when 10 β -hydroxy-norethisterone was transformed by *C. simplex* into 10 β ,17 β -dihydroxy-17-ethynyl-1,4-estradien-3-one (III; mp. 265–267°C; $[\alpha]_D - 79^\circ$ ($c = 1$, methanol)). The structure of III was established spectroscopically. The UV spectrum ($\lambda_{\max}$ 241 nm) and the IR spectrum ($\nu_{\text{C=O}}$ 1665, $\nu_{\text{C=C}}$ 1615, 1605 cm^{-1}) were consistent with the assumed structure of ring A in III. The signals of olefinic protons in the PMR spectrum of III ($\delta_{\text{H-1}}$ 7.2, $\delta_{\text{H-2}}$ 6.1, $\delta_{\text{H-4}}$ 5.95 ppm; $J_{1,2} = 10$, $J_{2,4} = 2$, $J_{1,4} \sim 0$ Hz; DMSO- d_6) were characteristic of the $\Delta^{1,4}$ -3-keto-structure. In the mass spectrum of III, the molecular ion (M^{+}) appeared at m/e 312 indicating the loss of two hydrogen atoms during transformation.

Several fungi were able to hydroxylate norethisterone at position 6 β [4]. The structure of 6 β -hydroxy-norethisterone (IV; mp. 193–196°C; $[\alpha]_D - 123^\circ$ ($c = 1$, methanol)) was proved in the following way (Fig. 1). Jones oxidation

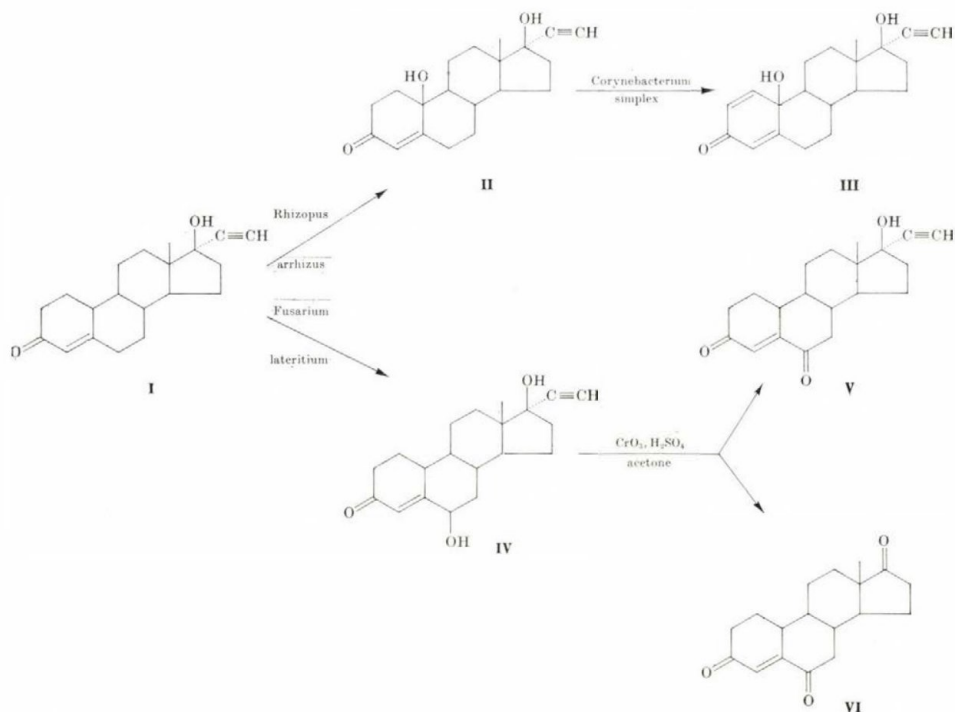


Fig. 1. Microbiological preparation of 6 β -hydroxy- and 10 β -hydroxy-derivatives of norethisterone

[7] of IV resulted in the formation of 17-ethynyl-17 β -hydroxy-4-estrene-3,6-dione (V; mp. 210–214°C; $[\alpha]_D -142^\circ$ ($c=1$, methanol)) as the main product. The UV spectrum of V ($\lambda_{\max}$ 253 nm) agreed with its Δ^4 -3,6-diketo-structure [8]. Carbonyl bands ($\nu_{C=O}$ 1692, 1672 cm^{-1}) in the IR spectrum of V indicated that the newly formed keto group was conjugated with the Δ^4 -double bond. The mass spectrum of V showed a molecular ion at m/e 312 and an abundant ion at m/e 123 corresponding to the characteristic ion of Δ^4 -3,6-diketo-steroids [9]. Chromic oxidation of IV also gave a small amount of the known 4-estrene-3,6,17-trione (VI) [8]. In the PMR spectrum of IV the magnitude of

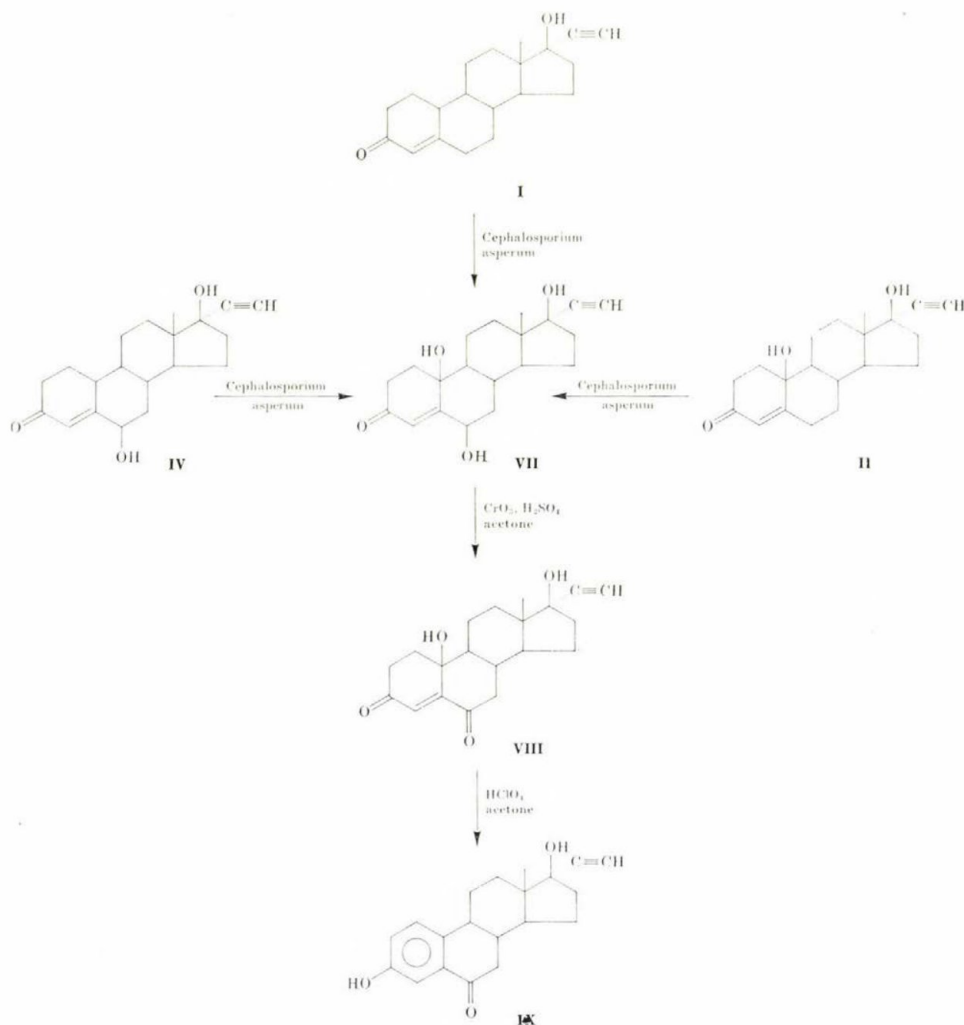


Fig. 2. Microbiological preparation and structural elucidation of 6 β ,10 β -dihydroxy-norethisterone

the coupling constants of the proton located at C-6 indicated that it has an equatorial orientation ($\delta\text{H-6}$ 4.4 ppm, t; $J = 3$ Hz; CDCl_3). Consequently, the hydroxyl group is in the axial 6β -position.

The formation of II and IV represents monohydroxylations on the activated allylic carbon atoms of norethisterone.

These processes also occurred in combination and yielded $6\beta,10\beta$ -dihydroxy-norethisterone (VII; mp. $201\text{--}204^\circ\text{C}$; $[\alpha]_{\text{D}} -69^\circ$ ($c = 1$, methanol)). The structure of VII was deduced from the fact that *C. asperum* produced this dihydroxy-derivative not only from norethisterone, but also from 6β -hydroxy-norethisterone as well as from 10β -hydroxy-norethisterone (Fig. 2); the structure was then corroborated chemically. The 6β -hydroxyl function was oxidized to ketone, then the 10β -hydroxyl group was split off with perchloric acid in acetone. The product obtained (IX) proved to be identical with the known 17-ethynyl-3,17 β -dihydroxy-1,3,5(10)-estratrien-6-one [10].

10β -Hydroxylation can also be accompanied by 11β -hydroxylation resulting in the formation of $10\beta,11\beta$ -dihydroxy-norethisterone (X; mp. $274\text{--}278^\circ\text{C}$; $[\alpha]_{\text{D}} +8^\circ$ ($c = 1$, methanol)) (Fig. 3).

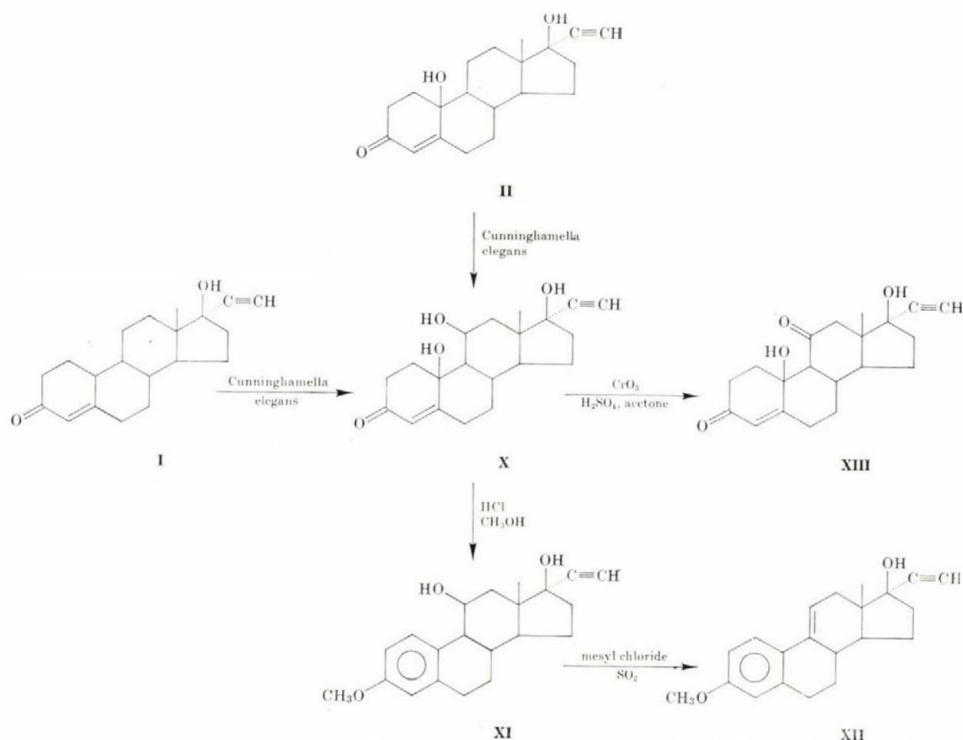


Fig. 3. Microbiological preparation and structural elucidation of $10\beta,11\beta$ -dihydroxy-norethisterone

The fact that *C. elegans* produced this dihydroxy compound from 10β -hydroxy-norethisterone, too, revealed the presence of a 10β -hydroxyl group in X. Elimination of the 10β -hydroxyl group from X with methanolic HCl afforded a monohydroxylated derivative of 17-ethynyl-3-methoxy-1,3,5(10)-estratrien-17 β -ol (XI, mp. 177–179°C; $[\alpha]_D^{25} +78.5^\circ$ (c = 1, chloroform)). By splitting off from XI the other microbiologically introduced hydroxyl group using the

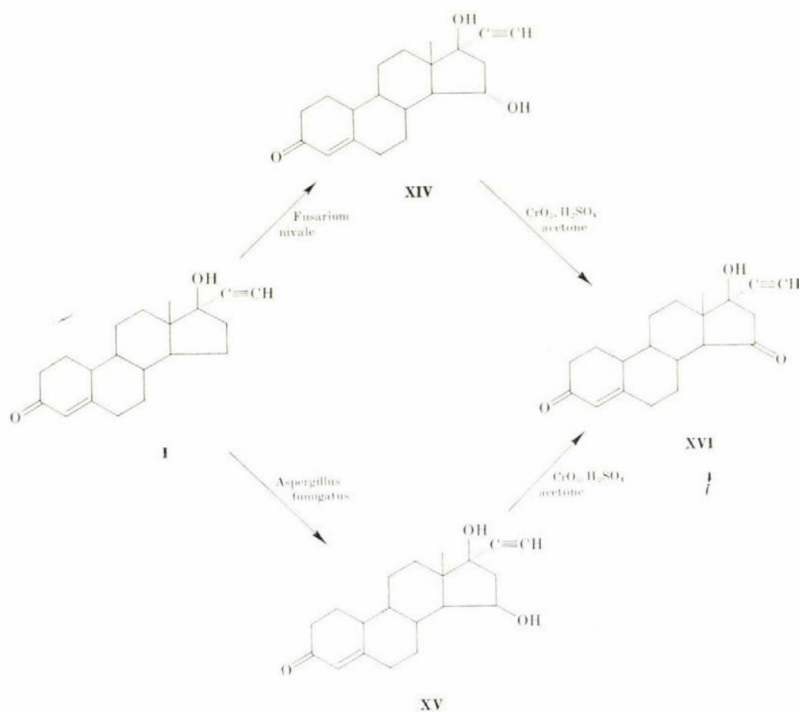


Fig. 4. Hydroxylation of norethisterone at positions 15α and 15β

method of HAZEN and ROSENBERG [11] we obtained a compound (XII) identical with the authentic 17-ethynyl-3-methoxy-1,3,5(10),9(11)-estratetraen-17 β -ol. The IR spectrum of compound XIII formed upon Jones oxidation of X showed an additional carbonyl band at 1690 cm^{-1} , indicative of a hydrogen-bonded oxo group located on one of the six-membered rings. Compared to the PMR spectrum of norethisterone, only a small change was observed in the chemical shift of C-18-methyl protons of XIII, excluding the C-12 position of the new oxo group in compound XIII [12]. From the above data it has been concluded that the secondary hydroxyl group of X was situated at C-11. As the signal of C-11-H in the PMR spectrum of X has a shape corresponding to an equatorial orientation, the hydroxyl group is in the axial 11β -position.

Another non-allylic monohydroxylation was observed in the ring A of norethisterone. As shown in Fig. 6, *A. kiliense* converted norethisterone into its 1 α -hydroxy-derivative (XVII; mp. 208–211°C; $[\alpha]_D -46^\circ$ (c = 1, methanol)) and to a lesser extent into II.

The fact that both XVII and II gave 17-ethynyl-3-methoxy-1,3,5(10)-estratrien-17 β -ol (XVIII) upon treatment with methanolic HCl, suggested that

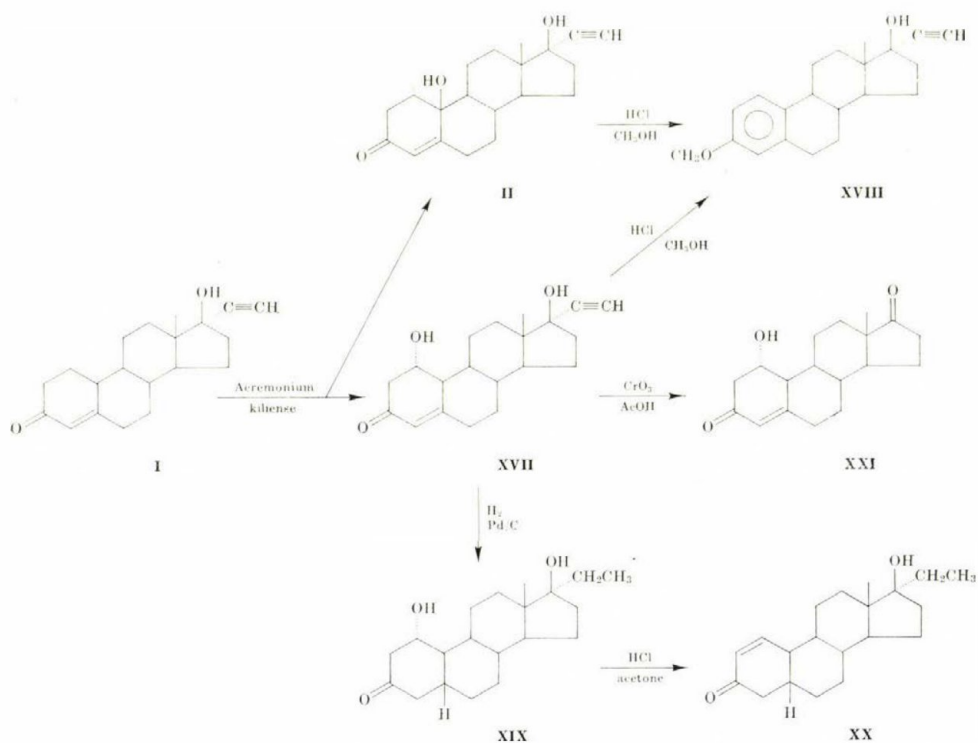


Fig. 6. Microbiological preparation and structural elucidation of 1 α -hydroxy-norethisterone

the new hydroxyl group of XVII was in ring A. Hydrogenation of XVII gave the 5 β -saturated derivative XIX. Elimination of the microbiologically introduced hydroxyl group from XIX with HCl in acetone afforded 17-ethyl-17 β -hydroxy-5 β -estr-1-en-3-one (XX). The structure of XX was derived from the following spectroscopical data. Its UV spectrum ($\lambda_{\max}$ 231 nm) and its IR spectrum ($\nu_{\text{C=O}}$ 1665, $\nu_{\text{C=C}}$ 1605 cm^{-1}) suggested the presence of a Δ^1 -3-one structure. The olefinic region of the PMR spectrum of XX ($\delta_{\text{H-1}}$ 7.18 ppm, dd, $\delta_{\text{H-2}}$ 6.0 ppm, d; $J_{1,2} = 10$, $J_{1,10} = 5.5$ Hz; CDCl_3) was characteristic of 5 β - Δ^1 -3-keto-19-norsteroids [13]. Location of the hydroxyl group on C-1 in XVII was proved by the fact that the proton geminal to the hydroxyl group

was coupled with three vicinal protons. Chromic oxidation of XVII in acetic acid afforded a 1-hydroxy-4-estrene-3,17-dione (XXI; mp. 224–227°C; $[\alpha]_D^{25} + 115^\circ$ ($c = 1$, chloroform)) which was different from the known 1 β -hydroxy-4-estrene-3,17-dione [14]. Direct evidence for the 1 α -configuration of the micro-biologically introduced hydroxyl group in XVII was obtained by PMR, ORD and CD investigations [15].

Preparation of 1 β -hydroxy and 11 β -hydroxy-norethisterone by *Botryodiplodia malorum* has recently been reported by GREENSPAN *et al.* [16].

Examination of the biological properties of the hydroxylated norethisterone derivatives isolated by us showed that 1 α -hydroxy-norethisterone was an effective postovulatory contraceptive agent [17].

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PROPERTIES OF HYDROXYSTEROID OXIDOREDUCTASE ISOLATED FROM YEAST

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Summary. Yeasts can advantageously be utilized for the production of the 17 β -hydroxy-derivative, from 3-methoxy-8,14-seco-1,3,5(10),9(11)-estratetraene-14,17-dione (14,17-dione) while 14 α -hydroxy and 14 α ,17 β -dihydroxy-derivatives are also formed. The biochemical properties of yeasts' enzymes responsible for the formation of the two monohydroxy-derivatives have been studied in detail. In the cell-free extract of *Saccharomyces* the presence of two hydroxysteroid oxidoreductases could be detected. The first enzyme forms 3 β ,17 β -dihydroxy-derivative from 5 α -androstane-3,17-dione. This enzyme is responsible for the formation of 17 β -hydroxy-derivative from 14,17-dione. The second enzyme forms 3 α -hydroxy-derivative from 5 β -androstane-3,17-dione as well as 14 α -hydroxy-derivative from its 14,17-dione. The cofactor of both enzymes is pyridine nucleotide. The two enzymes possessing different properties can selectively be inhibited.

Fourteen years ago it has been demonstrated that yeast cells could reduce steroid compounds stereospecifically. MAMOLI and VERCELLONE [1] in 1937 described the transformation of 4-androstane-3,17-dione to testosterone by baker's yeast. During many years this enzyme reaction seemed unimportant in the transformation of steroids until a few years ago KOSMOL *et al.* [2] have described the usefulness of a stereospecific reduction connected with the total synthesis of the 19-norsteroid skeleton. At that time the total synthesis of racemic norsteroids seemed to be an economical industrial process. The above authors reported that the cultures of different yeasts can advantageously be utilized for the production of 17 β -hydroxy-derivative (3-methoxy-8,14-seco-17 β -hydroxy-1,3,5(10),9(11)-estratetraen-14-one), from 14,17-dione (3-methoxy-8,14-seco-1,3,5(10),9(11)-estratetraene-14,17-dione), while some other microorganisms for the production of 14 α -ol-17-one (3-methoxy-8,14-seco-14 α -hydroxy-1,3,5(10),9(11)-estratetraen-17-one). One of these monoreduced compounds, 17 β -ol-14-one, proved to be an excellent key intermediate for the synthesis of optically active norsteroids.

The formation of these compounds was studied in detail using *Saccharomyces uvarum* and other microorganisms. *S. uvarum* according to KOSMOL *et al.* [2] forms two monoreduced and one bireduced compound from 14,17-dione but the main product is the 17 β -hydroxy-derivative (Fig. 1). The question was whether the different reduced products were formed by different specific enzymes or by one enzyme only, having less specificity.

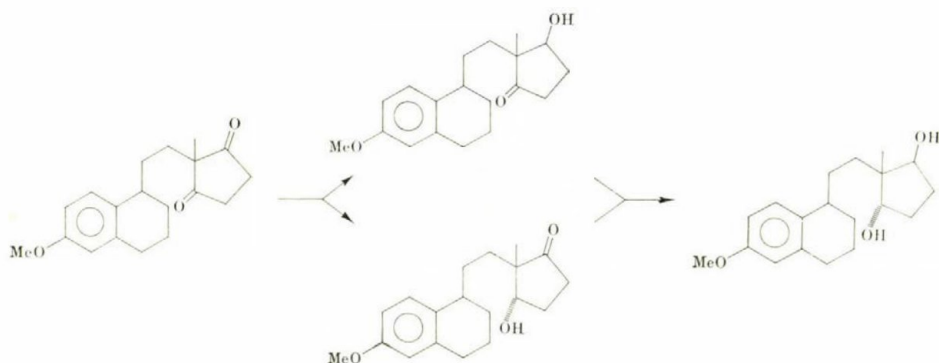


Fig. 1. Transformation of 3-methoxy-8,14-seco-1,3,5(10),9(11)-estratetraene-14,17-dione by *S. uvarum*

Materials and methods

Two strains were chosen; one of them was *S. uvarum* used by KOSMOL *et al.* [2] to produce 17 β -ol-14-one, and the other *Saccharomyces drosophilae* which formed 14 α -ol-17-one as the main product. The yeast cells were cultivated on a carbohydrate-rich medium, harvested by centrifugation, washed with tap water and suspended in 0.1 M phosphate buffer (pH 6.8). The substrate was added to the cell suspension by solving dimethylformamide with an appropriate quantity of ethanol. The transformation of compounds was followed by assaying the substrate and the products by thin-layer chromatography. Cell-free protein solution was obtained by means of a Braun Melsungen cell homogenizer and the cell debris was removed by centrifugation (50 000 g). The reaction mixture contained 1 mg 14,16-dione, 5 mg NADH₂ or NADPH, 5 mg protein in 1 ml phosphate buffer (0.1 M, pH 7.0) and was shaken at 28°C. The formation of hydroxyketones was detected by thin-layer chromatography.

Results and discussion

S. uvarum produced 17 β -ol-14-one and a small amount of 14 α -ol-17-one (Fig. 2). During the fermentation process, 14 α -ol-17-one is transformed into 14 α ,17 β -dihydroxy compound. It is apparent that the bisubstituted cyclopentane-dione is a more appropriate substrate for the yeast by hydroxysteroid oxidoreductase than the first reaction product, hydroxyketone. The rate of transformation of 14,17-dione was higher than with any other kind of natural steroid as substrate. The reducing activity was, however, very low when 14 α -ol-17-one was used as substrate. Transformation of 17 β -ol-14-one into 14 α ,17 β -dihydroxy-derivative could not be detected. The substrate could be reduced only in the presence of ethanol or isopropanol. In the absence of some oxidizable compounds, no reducing reaction, whereas in the absence of ethanol, formation of 14,17-dione from 17 β -ol-14-one was detectable. Fig. 3 illustrates the transformation of *S. drosophilae* which forms 14 α -ol-17-one as main product, thus the above two strains represent two entirely different groups of the yeasts selected. Many microorganisms were tested for reducing activity; the

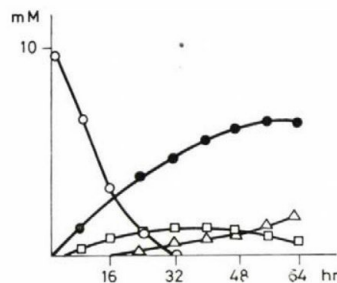


Fig. 2. Transformation of 3-methoxy-8,14-seco-1,3,5(10),9(11)-estratetraene-14,17-dione by *S. uvarum*. Reaction mixture: 2 mg yeast cell; 10 μ moles steroid compound; 10 μ l ethanol in 1 ml 0.1 M sodium phosphate buffer pH 6.8, shaken at 28°C. ○—○ 3-methoxy-8,14-seco-1,3,5(10),9(11)-estratetraene-14,17-dione; ●—● 3-methoxy-17 β -hydroxy-8,14-seco-1,3,5(10),9(11)-estratetraen-14-one; □—□ 3-methoxy-14 α -hydroxy-8,14-seco-1,3,5(10),9(11)-estratetraen-17-one; △—△ 3-methoxy-8,14-seco-1,3,5(10),9(11)-estratetraene-14 α ,17 β -diol

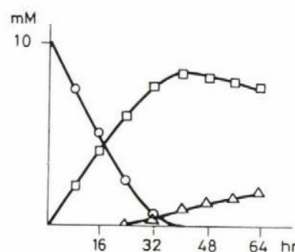


Fig. 3. Transformation of 3-methoxy-8,14-seco-1,3,5(10),9(11)-estratetraene-14,17-dione by *S. drosophilum*. Reaction mixture: 2 mg yeast cell; 10 μ moles steroid compound; 10 μ l ethanol in 1 ml 0.1 M sodium phosphate buffer pH 6.8, shaken at 28°C. ○—○ 3-methoxy-8,14-seco-1,3,5(10),9(11)-estratetraene-14,17-dione; □—□ 3-methoxy-14 α -hydroxy-8,14-seco-1,3,5(10),9(11)-estratetraen-17-one; △—△ 3-methoxy-8,14-seco-1,3,5(10),9(11)-estratetraene-14 α ,17 β -diol

majority reduced both oxo groups of 14,17-dione, but usually 14 α -ol-17-one was formed in larger amount than 17 β -ol-14-one.

The transformation of some kinds of natural steroid was subsequently investigated, since it was evident that 14,17-dione is not a natural substrate. Both 5 α and 5 β -androstane-3,17-dione were found to be appropriate substrates, as seen in Figs 4 and 5.

From each androstanedione three monoreduced and two bireduced derivatives were formed. The width of the arrow is proportional to the amount of derivatives formed in one hour. The reduced products were assayed by thin-layer chromatography, using authentic standards.

From the results obtained with *S. uvarum* the following conclusions have been drawn (Fig. 4). In the 5 α -series, the production of β -hydroxy-derivatives (3 β or 17 β) was faster than the formation of 3 α -hydroxy-compounds. In

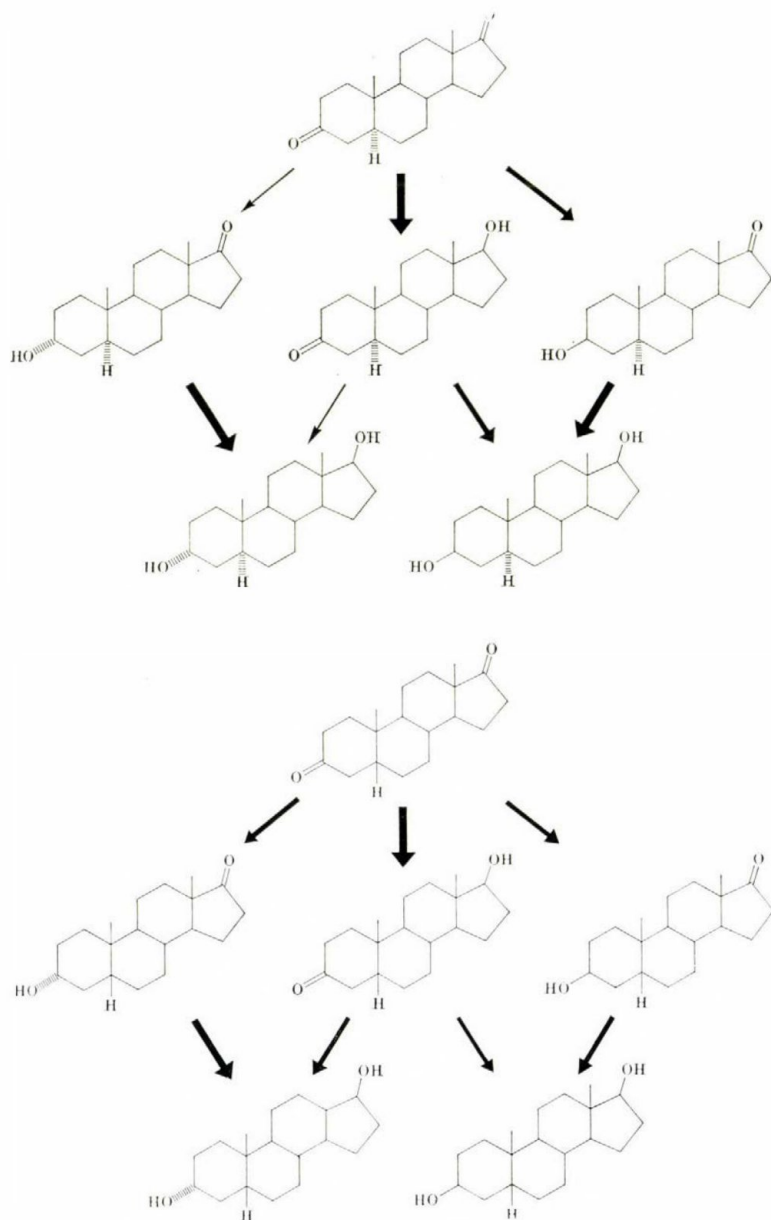


Fig. 4. Transformation of 5 α and 5 β -androstane-3,17-dione by *S. uvarum*

the 5 β -series, the formation of 3 α -hydroxy-compounds increased while the amount of β -hydroxy-derivatives decreased.

In the case of *S. drosophilum*, the reducing activity was reversed (Fig. 5). In the 5 α -series, much less β -hydroxy-compounds than 3 α -hydroxy-deriva-

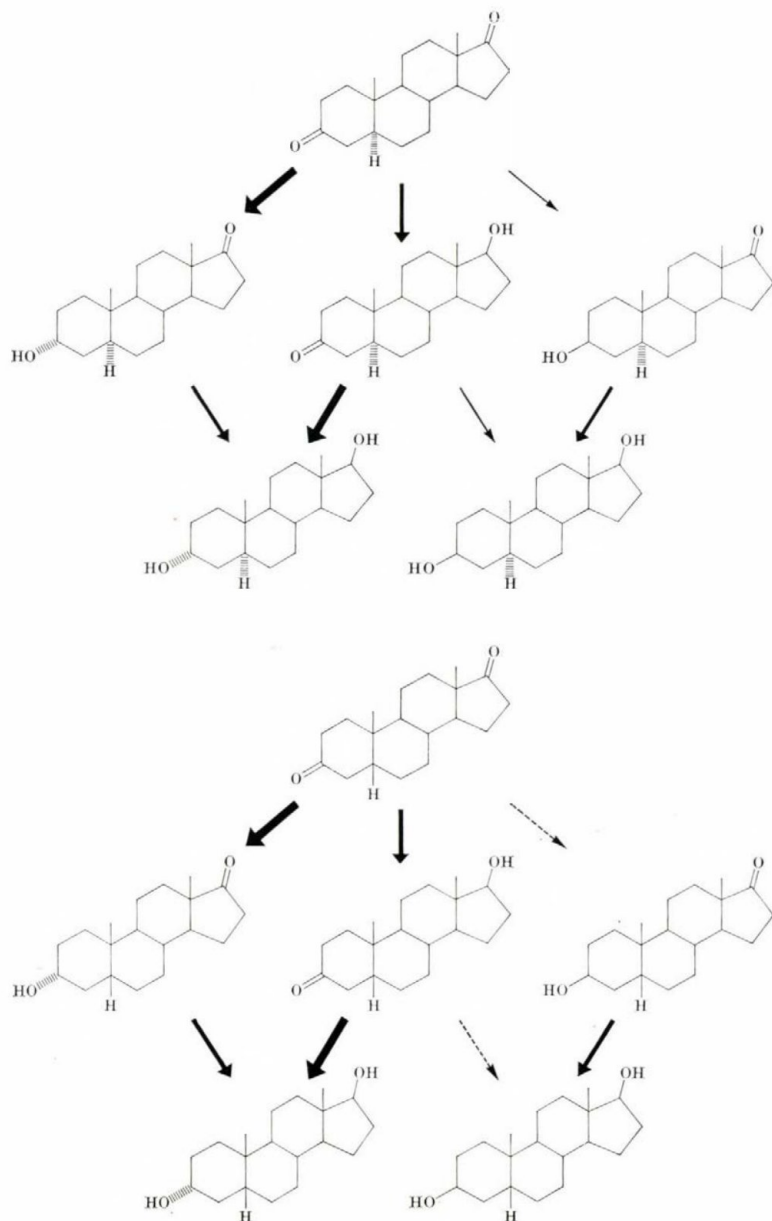


Fig. 5. Transformation of 5 α and 5 β -androstane-3,17-dione by *S. drosophilum*

tives were formed. In the 5β -series the formation of 3α -hydroxy-derivatives was faster than in the former case but traces of some reduced compounds with β -hydroxyl group could be detected.

These data suggest that there are two separate hydroxysteroid oxidoreductases in the yeast cells. One of them is 3α -hydroxysteroid oxidoreductase; its activity is high in *S. drosophilum* and can transform $14,17$ -dione to 14α -ol- 17 -one. The structure of the 5β -series proved to be more appropriate for this enzyme than those belonging to the 5α -series. The other enzyme is $3\beta(17\beta)$ -hydroxysteroid oxidoreductase which can form 17β -ol- 14 -one in *S. uvarum*. For this enzyme steroid compounds of the 5α -series were found to be the most appropriate substrates.

The assumption that 3α -hydroxysteroid oxidoreductase catalyzes the formation of 14α -ol- 17 -one, whereas $3\beta(17\beta)$ -hydroxysteroid oxidoreductase that of 17β -ol- 14 -one, was verified by experiments employing estrone as substrate. Fig. 5 illustrates that estradiol was formed from estrone by cells of *S. uvarum*, but when *S. drosophilum* was used, the compound could be detected in traces only (Fig. 6).

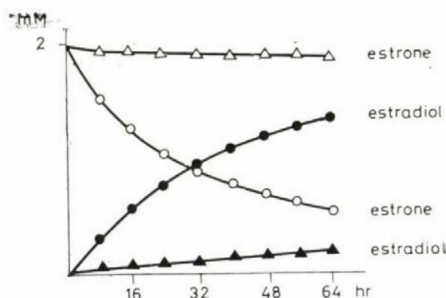


Fig. 6. Transformation of estrone by yeasts. Reaction mixture: 2 mg yeast cell, 10 μ moles steroid compound, 10 μ l ethanol in 1 ml 0.1 M sodium phosphate buffer pH 6.8, shaken at 28°C. ○—○, ●—● assay with *S. uvarum*; Δ—Δ, ▲—▲ assay with *S. drosophilum*

Concerning the theory of two different enzymes, we have succeeded in finding some compounds which selectively inhibited one of the two oxidoreductases. β -Naphthol decreases the activity of 3α -oxidoreductase as indicated in Fig. 7 demonstrating the reducing activity of *S. uvarum* on androstenedione in the presence and absence of inhibitor. In the presence of β -naphthol, 3α -hydroxyl group was formed in traces only (Fig. 7).

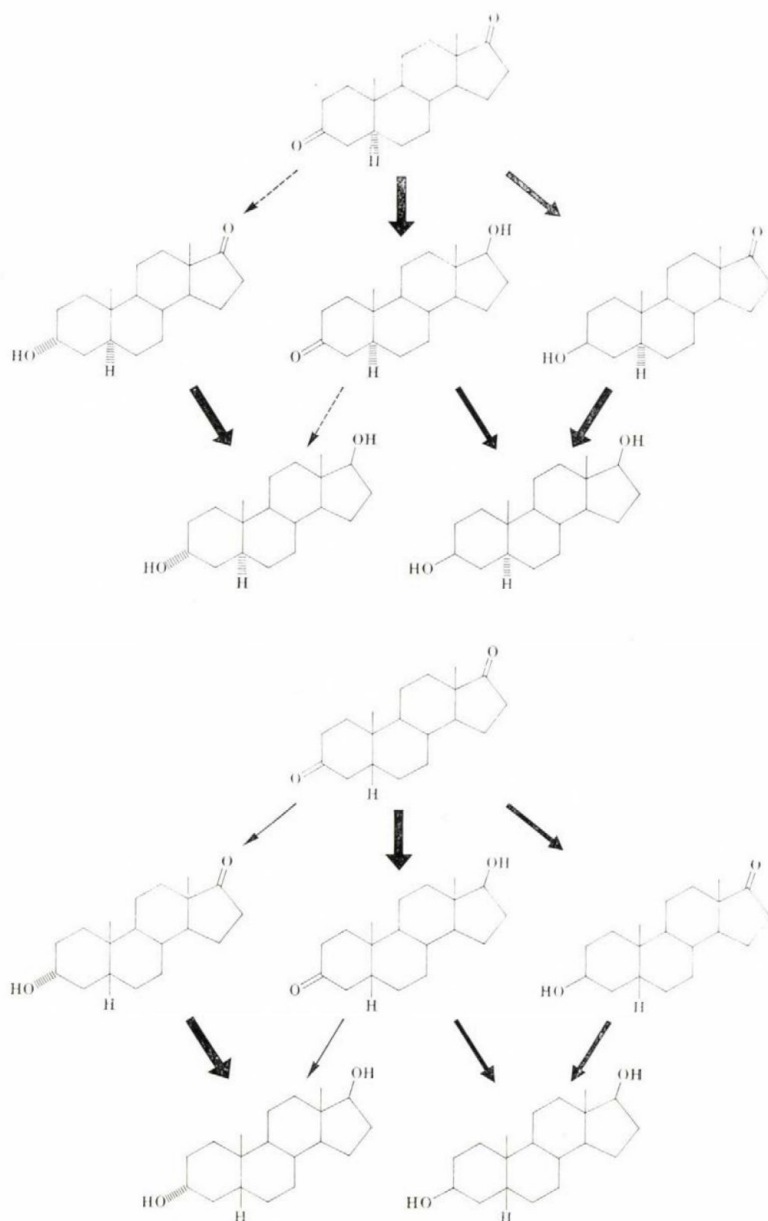


Fig. 7. Transformation of 5α - and 5β -androstane-3,17-dione by *S. uvarum* in the presence and absence of β -naphthol

The effect of β -naphthol was useful in the transformation of 14,17-dione. Quenching the formation of 14 α -ol-17-one, the yield of 17 β -ol-14-one increased in the presence of the compound (Fig. 8).

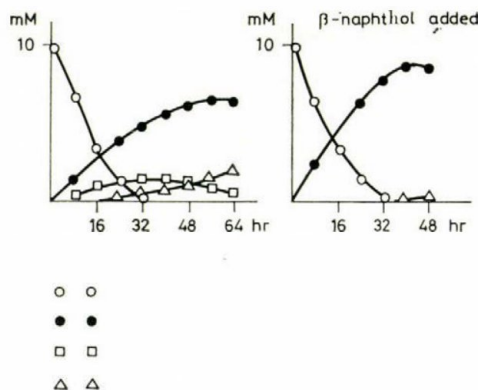


Fig. 8. Transformation of 3-methoxy-8,14-seco-1,3,5(10),9(11)-estratetraene-14,17-dione by *S. uvarum*. Reaction mixture: 2 mg yeast cell, 10 μ moles steroid compound, 10 μ l ethanol in 1 ml 0.1 M sodium phosphate buffer pH 6.8, shaken at 28°C. ○—○ 3-methoxy-8,14-seco-1,3,5(10),9(11)-estratetraene-14,17-dione; ●—● 3-methoxy-17 β -hydroxy-8,14-seco-1,3,5(10),9(11)-estratetraen-14-one; □—□ 3-methoxy-14 α -hydroxy-8,14-seco-1,3,5(10),9(11)-estratetraen-17-one; △—△ 3-methoxy-8,14-seco-1,3,5(10),9(11)-estratetraene-14 α ,17 β -diol

Both hydroxyketones could be formed from 14,17-dione in cell-free system. Using cell-free extracts of *S. uvarum*, the amount of 14 α -ol-17-one and 17 β -ol-14-one was not proportional to the amount of both hydroxyketones produced by whole cells. The 3 β (17 β)-hydroxysteroid oxidoreductase might be more sensitive than 3 α -hydroxysteroid oxidoreductase. The addition of β -naphthol to the reaction mixture suppressed, however, the formation of 3 α -hydroxysteroid oxidoreductase, and only 17 β -ol-14-one was formed.

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PRODUCTION OF 13 β -ALKYL-3-METHOXY-8,14-SECO-1,3,5(10),9(11)-GONATETRAEN-14 β -OL-17-ONES AND 13 β -ALKYL-3-METHOXY-8,14-SECO-1,3,5(10),9(11)-GONATETRAEN-17 α -OL-14-ONES BY MICROBIAL ENZYMES

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Summary. For the purpose of producing hydroxy-keto-seco-steroids in which hydroxyl group is attached to a carbon atom having the R-configuration, numerous biochemically active microorganisms were tested without any success. The hydroxysteroid oxidoreductase enzymes of the investigated bacterial, yeast and fungal strains were suitable only for the production of 17 β -ol-14-one and 14 α -ol-17-one derivatives. The required compounds were prepared by combinations of enzymatic reactions with chemical reduction. (i) By hydroxysteroid oxidoreductase of *Saccharomyces uvarum* and *Saccharomyces drosophilum*, 17 β -ol-14-one and 14 α -ol-17-one derivatives of 14,17-dione, respectively, were obtained. (ii) The above compounds were acetylated then reduced by sodium borohydride. (iii) 14 β ,17 β -diol-17-acetate and 14 α ,17 α -diol-14-acetate were dehydrogenated by hydroxysteroid oxidoreductase of *Nocardia* sp. and *Mycobacterium* sp., respectively, in the presence of steroid esterase. The reaction mixture contained either 14 β -ol-17-one or 17 α -ol-14-one derivatives, since oxidation by hydroxysteroid oxidoreductase was limited to the hydroxyl group attached to a carbon atom having the S-configuration.

For the synthesis of *ent*-estrane, the most appropriate intermediate is 3-methoxy-14 β -hydroxy-8,14-seco-1,3,5(10),9(11)-estratetraen-17-one (14 β -ol-17-one). This could be verified by the total synthesis of 1-estrone from this hydroxyketone [1].

While 17 β -ol-14-one, the key intermediate of estrone synthesis, could be formed from 3-methoxy-8,14-seco-1,3,5(10),9(11)-estratetraene-14,17-dione (14, 17-dione) by a microbiological process according to KOSMOL *et al.* [2], a direct method for the production of 14 β -ol-17-one was lacking.

Materials and methods

Several hundred microorganisms were screened by a simple method. The bacteria were cultured in shaker flasks containing yeast extract-peptone medium. Yeasts were grown on a submerged culture containing cornsteep-liquor and sugar. The substrate was added into the culture in dimethylformamide solution. After incubation the whole quantity of steroids was extracted with dichloromethane which was then removed and subsequently the sample was dried over sodium sulphate, applied on thin-layer plates and chromatographed with appropriate standards. As a solvent, chloroform and methanol (95 : 5) of analytical grade was used. The spots were detected by alcoholic sulphuric acid at 105°C. The optical rotation power of the obtained products was also recorded.

From the bacterial cells, cell-free extracts were obtained by using an X-press and a MSE High Speed 25 centrifuge. Activity of α and β enzymes was estimated according to MARCUS and TALALAY [3].

Results and discussion

During the course of large-scale screening programmes aiming at the selection of appropriate microorganisms for the production of hydroxyketones with a good yield, we could isolate some yeasts and bacteria with a high level of hydroxysteroid oxidoreductase, but they were useful only to form 17β -ol-14-one or 14α -ol-17-one. Efforts have failed to find a direct method for producing all the four theoretically possible hydroxyketones separately. By chemical reduction, a mixture of hydroxyketone and dihydroxy compounds could be obtained (Fig. 1).

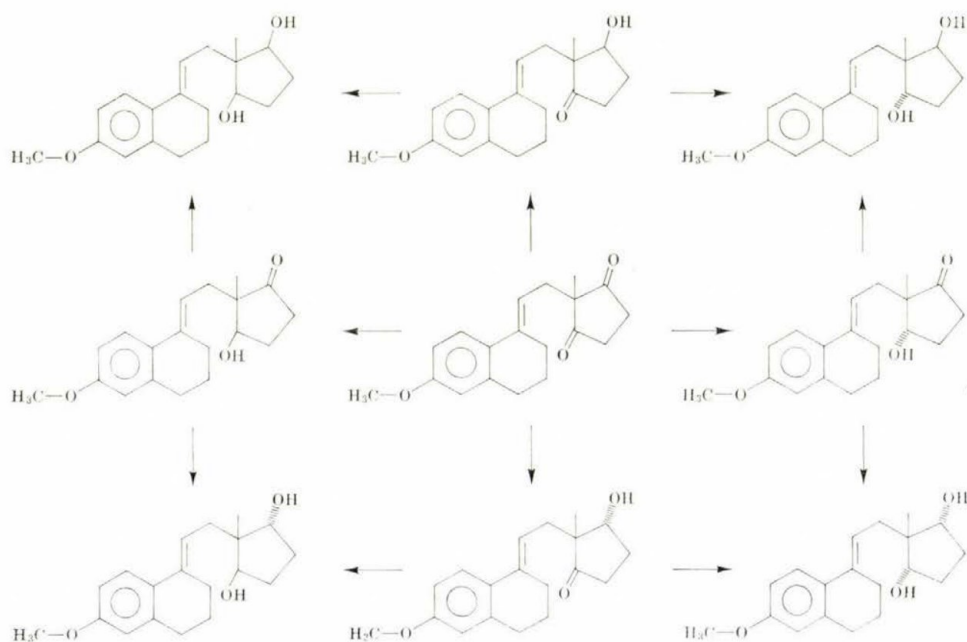


Fig. 1. Reduced products from 3-methoxy-8,14-seco-1,3,5(10),9(11)-estratetraene-14,17-dione

The various microorganisms tested could be classified in five different groups.

1. The substrate was metabolized practically completely.
2. Biochemical activity could not be detected.
3. A mixture of reduction products was detectable.
4. β -olone was mainly formed.
5. α -olone could mainly be detected.

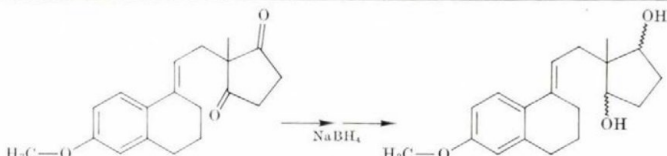
When the products were tested for optical rotation, it had to be ascertained that the hydroxysteroid oxidoreductase enzymes of the tested bacteria, yeasts and fungi were suitable for the sole production of 17β -ol-14-one or 14α -ol-17-one derivatives. In numerous cases the optical rotation showed that

α -olone was a mixture of 14 α -ol-17-one and of 17 α -ol-14-one. In our case 17 α -ol-14-one could not be detected as the main product, as described by Japanese authors [4].

We could never find any traces of the formation of 14 β -ol-17-one which seems to be one of the best intermediates for the production of *ent*-estranes. Efforts were made to obtain this compound with a good yield, and now we have succeeded in obtaining any kind of hydroxyketone by the combination of microbial and chemical methods.

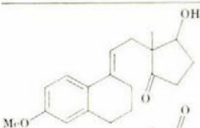
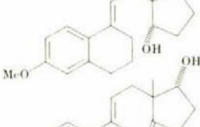
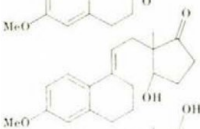
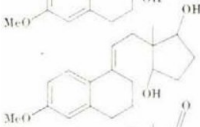
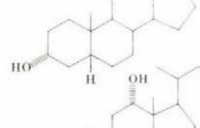
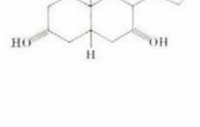
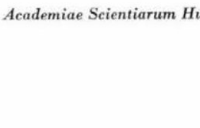
The combined method was based chiefly on the stereospecificity and reversibility of hydroxysteroid oxidoreductases. Theoretically, four hydroxyketones can be obtained by reducing the keto groups of 14,17-dione. Subsequent reduction of the two enantiomer pairs yielded four dihydroxy compounds: 14 α -ol-17-one, 17 α -ol-14-one, 14 β -ol-17-one and 17 β -ol-14-one. Two of them could be isolated as racemic compounds because their hydroxyl groups are in the *trans* position (14 α ,17 β -dihydroxy and 14 β ,17 α -dihydroxy derivatives). In the other two compounds the hydroxyl groups are in *cis* position but they differ from each other with respect to their relative steric position to the 13 β -alkyl chain (14 α ,17 α -dihydroxy and 14 β ,17 β -dihydroxy compounds). The chemical reduction by sodium borohydride could be directed by the steric effect of the groups, as indicated in Table I.

Table I
Chemical reduction by sodium borohydride

	
Substrate	Monoreduced derivatives
14,17-dione	85% 14 α -ol-17-one + 17 α -ol-14-one and 15% 14 β -ol-17-one + 17 β -ol-14-one
	Bireduced derivatives
17 β -ol-14-one	54% 14 β , 17 β -diol (<i>cis</i>) and 46% 14 α , 17 β -diol (<i>trans</i>)
14 α -ol-17-one	41% 14 α , 17 α -diol (<i>cis</i>) and 59% 14 α , 17 β -diol (<i>trans</i>)
17 β -ol-14-one 17-acetate	89% 14 β , 17 β -diol 17-acetate and 11% 14 α , 17 β -diol 17-acetate
14 α -ol-17-one 14-acetate	84% 14 α , 17 α -diol 14-acetate and 16% 14 α , 17 β -diol 14-acetate

In the first reduction the hydride ion could attack the coplanar ring easier from the β -side than from the α -side which has a larger substituent. The direction of the next reduction is influenced by the joint hindrance of 13 β -methyl

Table II
Activity of steroid dehydrogenases measured by NAD reduction at 340 nm/min

Substrate	<i>Flavobacterium dehydrogenans</i> crude extract	<i>Mycobacterium phlei</i> crude extract	<i>Nocardia</i> sp. Purified enzymes	
			Crude extract	α β
	005	042	036	005 080
	0501	05	043	076 006
	020	025	008	028 003
	000	003	002	002 001
	065	085	070	080 072
	075	062	035	090 000
	008	020	040	040 020
	005	068	080	008 098
	038	056	070	080 041
	006	081	070	013 110
	078	106	120	072 062
	122	110	100	102 008

and that of the hydroxyl group. The controlling effect of the hydroxyl group becomes predominant after acylation.

From these results it was obvious that by the reduction of optically active hydroxyketones (17β -ol-14-one or 14α -ol-17-one) mainly *cis*-hydroxy-derivatives produced by fermentation process were obtainable when *Saccharomyces uvarum* or *Saccharomyces drosophilum* was used. In these *cis*-diols, only those hydroxyl groups which had been formed by yeast could be dehydrogen-

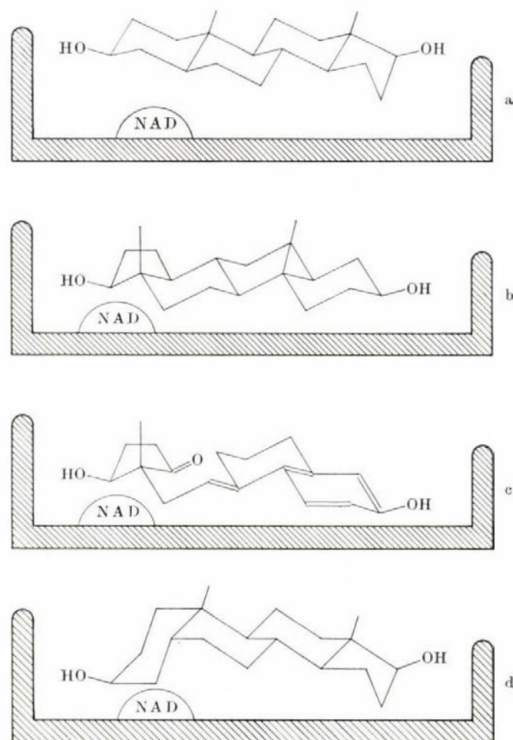


Fig. 2. Position of steroid compounds on the hypothetical surface of β -enzymes.
a, b = 5β -androstane- $3\beta,17\beta$ -diol; c = $3,17\beta$ -dihydroxy- $8,14$ -seco- $1,3,5(10),9(11)$ -estratetraen- 14 -one; d = 5β -androstane- $3\beta,17\beta$ -diol

ated by bacterial hydroxysteroid oxidoreductases. Three bacterial strains were selected from the above transformation process and the stereospecificity of their hydroxysteroid oxidoreductase was investigated. Experiments were carried out mostly in cell-free systems. NAD reduction was followed spectrophotometrically. Data obtained in these experiments and shown in Table II supply information concerning the structure of the enzyme surface surrounding the active centre.

Different kinds of hydroxyketones and dihydroxy derivatives of $14,17$ -dione were used as substrates but dehydrogenase activity was assayed in every

case also in the presence of 5α and 5β steroids. It is known that 14,17-dione is not a natural substrate for these enzymes.

In cell-free system, *Flavobacterium* oxidized 17α - and 14α -hydroxyl groups of compounds 14α -ol-17-one and 17α -ol-14-one, moreover, *Mycobacterium* and *Nocardia* oxidized all applied substrates with the exception of 14β -ol-17-one. The α - and β -hydroxysteroid oxidoreductase was separated from the crude extract of *Nocardia*. The substrate specificity of both fractions was similar to

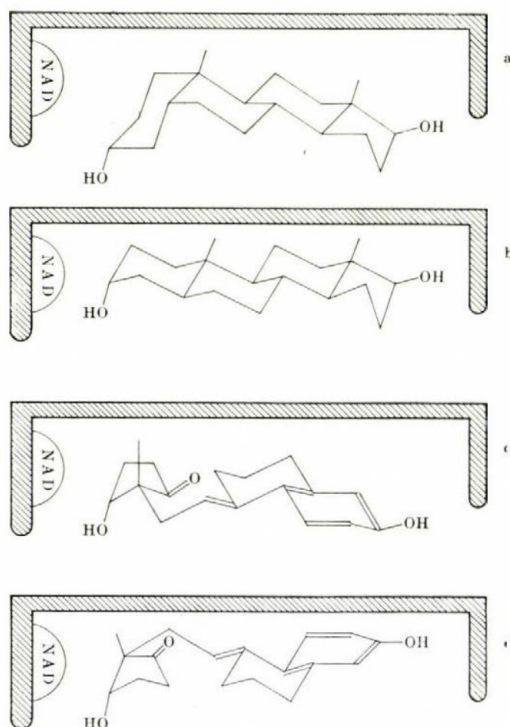


Fig. 3. Position of steroid compounds on the hypothetical surface of α enzymes. a = 5β -androstane- 3α , 17β -diol; b = 5α -androstane- 3α , 17β -diol; c = 3,17 α -dihydroxy-8,14-seco-1,3,5(10),9(11)-estratetraen-14-one; d = 3,14 α -dihydroxy-8,14-seco-1,3,5(10),9(11)-estratetraen-17-one

the one found by MARCUS and TALALAY [3] for wild type *Pseudomonas testosteroni*.

The conclusion from the experimental data must be drawn carefully because all of the reactions are reversible, and the product of the first reaction could serve as substrate for the second reaction. In living bacteria the direction of the reaction depends on the intermediary metabolism but in cell-free systems the reaction is directed mainly by the pH. Maximum rate of reduction could be measured at pH 6.5, whereas oxidation at pH 9.5.

The experimental data obtained in cell-free systems and in living cells allowed to conclude to the presence in the bacterial cells of two hydroxysteroid oxidoreductases. Both prefer the equatorial orientation of the hydroxyl group related to ring A, though the activity of the enzymes is influenced by the junction of rings A and B. The 14,17-dione molecule could be transformed by both the α and the β enzymes as it could mimick the conformation of natural steroid. Originally, bisubstituted cyclopentanedione has a coplanar structure and the enzymatic reduction is directed by its substituents. One of the prochiral

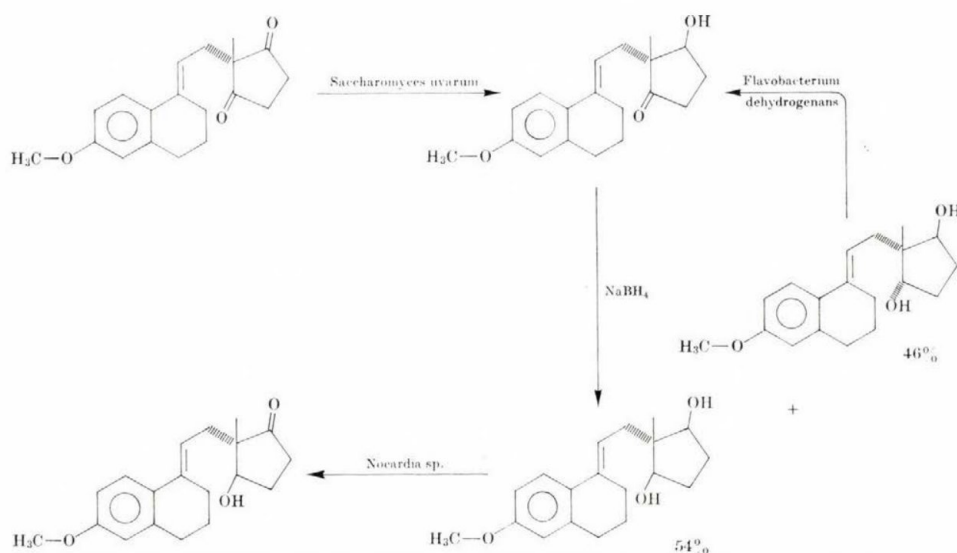


Fig. 4. Production of 3-methoxy-14 β -hydroxy-8,14-seco-1,3,5(10),9(11)-estratetraen-17-one. 1. 17 β -hydroxyketone produced by *S. uvarum*. 2. Production of *cis*-dihydroxy compound by chemical reduction and subsequent separation. 3. Selective oxidation by *Nocardia* sp. 4. Reoxidation of side-product by *Flavobacterium*

oxo groups (C17) could be reduced by the β -enzyme, while the other (C14) by the α -enzyme. As a substrate for the second reaction the first reduced product is less appropriate than the diketone (14,17-dione) was in the former case. Not only the coplanar character of ring D has changed but a new steric effect of the new hydroxyl group and the quasiaxial 13-methyl group has also to be reckoned with. Figs 2 and 3 demonstrate the way how the seco-estratetraene derivatives are mimicking the conformation of natural steroids. The position of the three carbon atoms is very important. They may be in position 2, 3, 4 on ring A or in position 16, 17, 13 on ring D. It is conceivable that at C17 only an oxo or a hydroxyl group of seco-derivative could find a site in the active centre of 3 β -oxidoreductase. It is hardly possible to place the C14 atom in the same position, though ring D can be rotated without restriction.

The enzyme prefers the equatorial orientation of hydroxyl group in 5 α -series because in that case NAD is situated under the substrate (Fig. 2). Of course, in the 5 β -series the enzyme oxidizes the axial 3 β -hydroxyl group

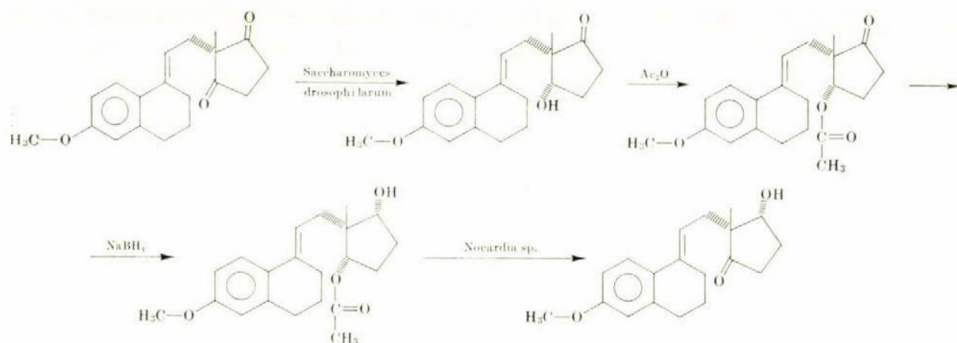


Fig. 5. Production of 3-methoxy-17 α -hydroxy-8,14-seco-1,3,5(10),9(11)-estratetraen-17-one. 1. 14 α -hydroxy-ketone produced by *S. drosophilum*. 2. Formation of acyl-derivatives. 3. Deacylation and selective reduction. 4. Selective reoxidation by *Nocardia* sp.

but this process is slow. In the case of 3 α -hydroxysteroid oxidoreductase, the position of the three carbon atoms (Nos 2, 3, 4) is fixed and NAD is situated under the plane of ring A (Fig. 3). In the 5 β -series the enzyme also prefers the equatorial orientation, but the axial one in the 5 α -series. Both the 14 α - and the 17 α -hydroxyl groups of seco-compounds can be oxidized by the enzyme.

When the 14 α -hydroxyl group is positioned in the active centre, the orientation of the whole molecule is similar to the 5 β -steroids.

Finally, Fig. 4 shows the scheme of production of 14 β -hydroxyketone, Fig. 5 that of 17 α -hydroxyketone and Fig. 6 demonstrates the scheme for the most economical production of 14 β -hydroxyketone.

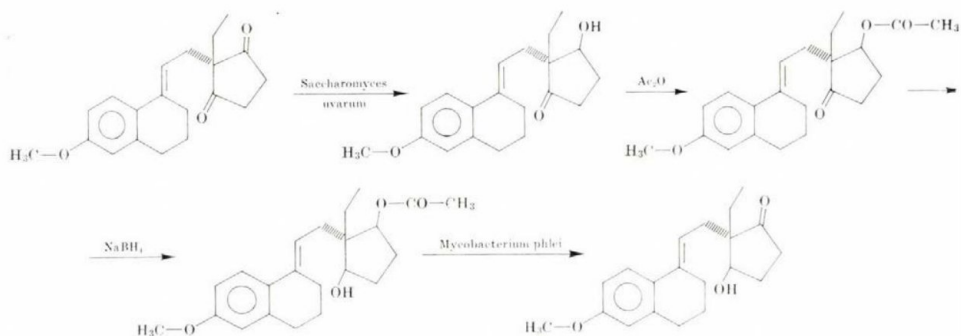


Fig. 6. Production of 3-methoxy-13 β -ethyl-14 β -hydroxy-8,14-seco-1,3,5(10),9(11)-gonatetraen-17-one. 1. 17 β -hydroxyketone production by *S. uvarum*. 2. Formation of acyl-derivatives. 3. Selective reduction. 4. Deacylation and selective reoxidation by *Mycobacterium*.

The activity of 17β -hydroxysteroid oxidoreductase was the same in *Mycobacterium* and *Nocardia* when 13β -methyl-dihydroxy compound was used as substrate, but when 13β -ethyl-dihydroxy compounds were used only transformation by *Mycobacterium* gave a good yield. The fact that *Mycobacterium* is the most useful strain for the transformation of 13β -ethyl homologues, could be verified in cell-free systems.

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CLEAVAGE OF CARDIAC STEROID GLUCOSIDES BY PURE β -GLUCOSIDASE FROM *ASPERGILLUS WENTII*

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Summary. Electrophoretically pure β -glucosidase (β -D-glucoside glucohydrolase, EC 3.2.1.21) A_3 from *Aspergillus wentii* was shown to deglucosylate cardiac steroid biosides, triosides and tetraosides with a terminal β -D-glucoside residue linked to the aglycon sugar in 1 $\rightarrow$ 6 or 1 $\rightarrow$ 4 position. In dependence on the structure and conformation of the aglycon, the deglucosylation rate may vary by six orders of magnitude.

Cardiac steroid biosides, triosides and tetraosides showing a terminal β -D-glucose residue in the sugar side chain, such as scillaren A, k-strophanthoside and lanatoside C are frequently used in the therapy of cardiac failure. The metabolic fate of such high molecular weight glucosides in the animal body is essentially determined by the fact that they are not degraded in liver and other metabolically active tissues but become deglucosylated in the gut by intestinal microorganisms [1–3].

Since several cardiac steroid glucosides were found to be resistant to β -glucosidase preparations of different origin but to be attacked by several microorganisms known to produce cellulases [1], the tentative conclusion was drawn that the deglucosylation in the gut was due to microbial enzymes of this type [1], although the glucoside type in question belongs rather to the substrate range of β -glucosidase.

To decide the problem of the enzyme involved, in the present paper 12 cardiac steroid glucosides were studied for their hydrolysis by the well-characterized pure β -glucosidase A_3 isolated from *Aspergillus wentii* [4].

Materials and methods

The cardiac steroid glucosides listed in Table I were paper chromatographically pure. As the water solubility of some compounds is rather limited, all steroid glucosides were primarily dissolved in propylene glycol. The solution was diluted immediately prior to use with buffer to a final concentration of 1% propylene glycol in the incubation mixture.

The β -glucosidase A_3 from *A. wentii* was a gift from Professor G. LEGLER and shown to be electrophoretically pure [4]. The enzymatic activity of the preparation amounted to 21–28 μ moles p-nitro-phenyl- β -D-glucoside cleaved per mg protein per min at a substrate concentration of 20 mM under the conditions described in Table I. Since the deglucosylation rate of the diverse cardiac steroid glucosides was found to vary within six orders of magnitude, the amount of the enzyme and the incubation time had to be adapted so as to meet the requirements

for the measurement of the initial rate. Thus, the incubation conditions were for compounds 1 and 2: 6–8 ng protein for 1 min; compounds 3–6, 10 and 12: 40 to 100 ng protein for 15 min; compounds 7–9 and 11: 80 to 1300 ng protein for 1–6 hr. The protein concentration was measured according to the procedure described by LEGLER [4].

The incubation was terminated by extraction of the steroid glycosides from the incubation mixture (2 ml) with 10 ml chloroform-ethanol 3 : 1 (v/v). The extracted deglucosylated products were separated and identified by paper chromatography (descending on Schleicher-Schüll 2043 bMg1) in the following solvent systems: chloroform : tetrahydrofuran (1 : 1)/formamide for metabolite of compound 1; butanol/H₂O for metabolite of compound 2; xylol : methylethylketone (1 : 1)/formamide for metabolites of compounds 3, 5, 7, 10 and 12; and xylol : methylethylketone (1 : 2)/formamide for metabolites of compounds 4, 6, 8, 9 and 10. Details of the procedures including the quantitative determination of the deglucosylated products have been described earlier [5–7]. Recovery of the various products of enzymatic degradation when carried through the whole procedure amounted to 80 to 100%. The results are corrected for these losses.

Incubation of the cardiac steroid glucosides with the buffer alone under otherwise identical conditions (cf. Table I) yielded no non-enzymatic degradation.

Results

Although the number of the steroid glucosides tested was limited and some representatives suitable for comparison were not available, the experimental data shown in Table I suggested the following conclusions concerning the substrate specificity of electrophoretically pure β -glucosidase A₃ from *A. wentii* [4].

The relative deglucosylation rate of the various compounds varied within six orders of magnitude. This might have been due to the structure of the steroid moiety, the length of the sugar side chain, the position of the linkage between the glucose residue and its aglycon, the structure of the aglycon, or/and the conformation of the glucose-containing bioside.

The constitution of the steroid moiety appears to be insignificant. Glucosides with the same steroidal part but different in their oligosaccharide components (cf. compounds 1 : 9 : 11; 2 : 5 : 6; 3 : 7 and 4 : 8) differed considerably in deglucosylation rate. With a "purified enzyme preparation" from *Aspergillus oryzae* GVOZDJAK and KOLESNIKOV [9] also found no influence of the steroid component on the deglucosylation rate of cardiac steroid biosides different in the steroidal component only.

The length of the sugar side chain seems to be of equally minor importance. Tetraosides and triosides are partially hydrolysed easier than biosides (cf. e.g. compounds 3 : 1 : 11). Surprisingly enough, the ratio of the deglucosylation rates of the glucosides with β -D-(1 $\rightarrow$ 6) and -(1 $\rightarrow$ 4) linkages is heavily in favour of the former (cf. 1–2 : 3 : 10), whereas the reverse ratio is known for β -glucosidase from sweet almonds, indeed in the latter cases with glucose as aglycon [8].

Thus, with the glucosides tested, the nature of the aglycon is of decisive importance for the rate of deglucosylation. The highest rates were found with glucose as aglycon. If the glucose was replaced by digitoxose, the rate was

Table I

*Cleavage of cardiac steroid glucosides by β -glucosidase A_3 from *A. wentii**

The various glucosides at a standard concentration of 0.1 mM were incubated with suitable amounts of the enzyme in 50 mM glycine/HCl buffer pH 3.5 at 35 °C for periods required for obtaining deglucosylation of the substrate between 20–50 mole%

Substrate		Deglucosylation rate μ moles deglucosylated compound/ mg enzyme protein $\times$ min	No. of experiments
Trivial name of cardiac steroid glucoside and its steroidal component (in brackets)	Constitution of sugar residue*		
1. Thevetin B (Digitoxigenin)	β -D-Glucose-(1 $\rightarrow$ 6)-D-glucose-L-thevetose-St	26 $\pm$ 1	6
2. k-Strophanthoside (Strophanthidin)	β -D-Glucose-(1 $\rightarrow$ 6)-D-glucose-D-cymarose-St	11 $\pm$ 2	5
3. Deacetyl-lanatoside C (Digoxigenin)	β -D-Glucose-(1 $\rightarrow$ 4)-(D-digitoxose) ₃ -St	0.12 $\pm$ 0.01	8
4. Deacetyl-lanatoside B (Gitoxigenin)	β -D-Glucose-(1 $\rightarrow$ 4)-(D-digitoxose) ₃ -St	0.11 $\pm$ 0.005	3
5. Erysimoside (Strophanthidin)	β -D-Glucose-(1 $\rightarrow$ 4)-D-digitoxose-St	0.28 $\pm$ 0.01	2
6. k-Strophanthin- β (Strophanthidin)	β -D-Glucose-(1 $\rightarrow$ 4)-D-cymarose-St	0.16 $\pm$ 0.01	2
7. Lanatoside C (Digoxigenin)	β -D-Glucose-(1 $\rightarrow$ 4)-O-Acetyl-D-Digitoxose-(D-Digitoxose) ₂ -St	0.0001 $\pm$ 0	2
8. Lanatoside B (Gitoxigenin)	β -D-Glucose-(1 $\rightarrow$ 4)-O-Acetyl-D-Digitoxose-(D-Digitoxose) ₂ -St	0.0003 $\pm$ 0	2
9. Lanatoside A (Digitoxigenin)	β -D-Glucose-(1 $\rightarrow$ 4)-O-Acetyl-D-Digitoxose-(D-Digitoxose) ₂ -St	0.0001 $\pm$ 0	2
10. Scillaren A (Scillarenin)	β -D-Glucose-(1 $\rightarrow$ 4)-L-rhamnose-St**	0.04 $\pm$ 0	2
11. Thevebioside (Digitoxigenin)	β -D-Glucose-(1 $\rightarrow$ 4)-L-thevetose-St**	0	2
12. Scillirosid (Scillirosidin)	β -D-Glucose-(1 $\rightarrow$ 3)-St	0.05 $\pm$ 0	2

* For references cf. [12]; St = steroid moiety.

** The linkage of glucose at C4 of the aglycon is uncertain; cf. [12].

reduced by two orders of magnitude (cf. compounds 1–2 : 3–5). It cannot be decided whether the difference was caused by the variation of the substituents at C-2, C-3 or C-6. Incidentally, with sweet-almond β -glucosidase and peculiar biosides a pronounced effect of even slight changes at C-2 and C-3 of the aglycon was also evident [8].

The effect of the derivation at C-3 OH of digitoxose depends on the nature of the substituent. Whereas methylation shows but a minor influence (cf. compounds 5 : 6), acetylation caused a decrease of the deglucosylation rate by about three orders of magnitude (cf. compounds 3–4 : 7–9). Different

factors may have had a role in this effect. Presumably, the bulky acetyl residue influences the conformation of the digitoxose residue and, what is even more important, the rotational freedom of the planes of the digitoxose and glucose rings around the axis of the glucosidic linkage. The resulting spatial orientation of these ring planes showing the thermodynamically favoured greatest distance between the acetyl and the likewise large hydroxymethyl substituent at C-5 of glucose could impede the formation of the enzyme substrate complex. Moreover, an electronic effect of the acetyl residue on the strength of the glucosidic linkage cannot be excluded. It is noteworthy that extracts of various strains of *Penicillium* and *Aspergillum* deglucosylate lanatoside A either not at all or at a lower rate compared to deacetyl lanatoside A [10] and that acid hydrolysis is also somewhat slower with the acetylated derivatives [cf. 12].

Although the basis for a strict comparison was lacking, it seemed that the affiliation of the aglycon to the D- or L-series was not decisive for the bioside to become hydrolysed (cf. compounds 5 : 10). Within the L-series of aglycons, too, the substituents at C-2 and possibly at C-3 determine the tendency to deglucosylation (cf. compounds 10 : 11).

Hydrolysis at a low rate occurred also when the aglycon was a steroid (compound 12). This reminds of the fact that in the leaves or seeds of the plant serving as the source of the steroid glucoside, a "specific enzyme" is often found which is able to hydrolyse the glucosidic link (for a review, see [12]). Recently, a steroid β -D-glucosidase has been found in animal tissues which accepts 3- β -D-glucosides of phenolic estrogens as substrates but does not show any hydrolytic activity toward cellobiose or gentiobiose [13].

The glycosidic linkages of D-digitoxose, D-cymarose, O-acetyl-D-digitoxose, L-rhamnose and L-thevetose (cf. compounds 1-11) were not attacked by β -glucosidase, thus confirming the well-known specificity of this enzyme type with respect to the sugar part of the substrate and its anomeric configuration.

Discussion

The data available do not allow a mechanistic interpretation of the huge variation in the hydrolysis by β -glucosidase of the various cardiac steroid glucosides. Due to the limited solubility of the compounds, it was not possible to determine the K_m and V_{max} values so that it remained undecided whether the variation was based on differences in affinity of the diverse glucosides to the active centre or/and in the relative stability of the glucosyl-enzyme intermediate supposed to be essential for hydrolysis [14]. Contrary to the common belief that β -glucosidase tolerates large structural variations in the aglycon part of the substrate [14], Table I shows that slight derivations may grossly determine the occurrence of enzymatic deglucosylation.

The most remarkable result of the present study was the finding that β -glucosidase that is known to attack simple disaccharides, was able to hydrolyse triosides and tetraosides with a bulky steroid lactone substituent. In fact, β -glucosidase from bitter almonds did not deglucosylate cardiac steroid glucosides but the involved unsubstituted biosides (for references see [1]). Moreover, the β -glucosidases occurring in animal tissues were also unable to degrade cardiac steroid glucosides. Thus, β -glucosidase A_3 from *A. wentii* appears to show an unusual capacity to accept glucosides of higher molecular weight as substrates, a property hitherto described for cellulases only. Apparently, the occurrence of microbial β -glucosidases of the A_3 type is responsible for the deglucosylation of cardiac steroid glucosides in the gut, a step so important for their metabolic fate [1-3].

Acknowledgement. We are indebted to Professor G. LEGLER for supplies of the enzyme preparation used in this study.

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PROPERTIES OF Δ^5 -3 β -HYDROXYSTEROID OXIDOREDUCTASE ISOLATED FROM STREPTOMYCES GRISEOCARNEUS

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Summary. Δ^5 -3 β -hydroxysteroid oxidoreductase was extracted in magnesium-containing Tris buffer from sonicated *Streptomyces griseocarneus* cells. The enzyme was partially purified (150 $\times$) by ion exchange chromatography and gel filtration following $(\text{NH}_4)_2\text{SO}_4$ fractionation. Upon gel filtration on Sephadex G-75 to G-200, the greatest part of the activity gave a peak in the fractionation range. The enzyme obtained from the gel yielded small enzyme molecules on repeated chromatography. A molecular weight of 32 to 36 000 was calculated for the activity appearing in the fractionation range of Sephadex G-75 to G-200. The enzyme is highly specific for the irreversible oxidation of the 3 β -hydroxyl group in steroids with a trans-anellated A : B ring system with either C5 or C6 double bond. Δ^5 -3-ketosteroids are converted into Δ^4 -3-ketosteroids at a high rate, but the isomerase activity cannot be separated from the oxidoreductase activity either by chromatography or by selective heat inactivation. NAD, NADP, FMN or FAD did not influence the activity, but the enzyme is inactive in the absence of molecular oxygen.

WIX *et al.* [1] reported that cultures of *Streptomyces griseocarneus* convert 3 β ,17 α ,21-trihydroxy-5-pregnen-20-one into 17 α ,21-dihydroxy-4-pregnene-3,20-dione even in the presence of water insoluble organic solvents. The properties of this enzyme were studied in detail.

Materials and methods

S. griseocarneus strain K 451 was used throughout. Cultures were inoculated with a washed spore suspension harvested from an agar culture that had been incubated aerobically for 1–3 weeks. Cultivation was done in a minimal medium (DP) at 28°C with rotary shaking for 72–75 hr. Medium: the supernatant of a boiled suspension of 2.0% soy flour and 0.5% CaCO_3 containing 0.1% $(\text{NH}_4)_2\text{SO}_4$, 1.0% KH_2PO_4 , 0.05% MgCl_2 (pH 7.0) and 3.0% glucose dispensed in 200 ml quantities in 500 ml Erlenmeyer flasks.

Results

S. griseocarneus cultivated in inorganic medium does not yield Δ^5 -3 β -hydroxysteroid oxidoreductase. It is not known which component(s) of soy induce enzyme production. Variation of the medium (high soy content), time of cultivation (68–80 hr) and fermentor volume (0.5–100 litres) showed that the techniques described ensure the best enzyme production.

The cells were harvested by centrifugation, washed twice with 0.1 M Tris buffer pH 7.15 containing 5 mM MgCl_2 and suspended in 5 ml of Tris buffer

per gram of wet cells. Cell disruption was performed by an MSE ultrasonic disintegrator (20 kc, 100 W maximum output) for 4–6 min at maximum output by ice cooling. All subsequent operations were carried out at 0–4°C. Large cell debris were removed by centrifugation at $15\,000\times g$ for 10 min. The supernatant was then centrifuged at $67\,000\times g$ (MSE High Speed 25) for 25 min and the resulting supernatant was used as a cell-free extract.

From the cell-free extract a concentrate was prepared by adding 60 g per 100 ml of $(\text{NH}_4)_2\text{SO}_4$ to precipitate the proteins. The precipitate was dissolved in a minimum volume of Tris buffer. By centrifugation at $67\,000\times g$ for 20 min a considerable part of the inactive material was removed with an insignificant loss of activity. Traces of $(\text{NH}_4)_2\text{SO}_4$ were removed by gel filtration on Sephadex G-25. Chromatography on QAE-Sephadex A-50 was then performed in Tris buffer. Under these conditions the majority of inactive protein was bound to the ion exchanger, while 90% of the enzyme was eluted from the column. The resulting solution was concentrated by dialysis against Ficoll and eventually gel filtered on Sephadex G-100 equilibrated with Tris buffer. All enzyme solutions were stored at -14°C .

Enzyme assays. The assays were linear with regard to time and enzyme concentration under the conditions described. Oxidoreductase activity was determined by a modification of the method of RUBIN *et al.* [2], using dehydroepiandrosterone (DHEA) as substrate. In 3.9 ml volumes of 0.1 M Tris buffer pH 7.15 four logarithmic dilutions were prepared of the enzyme preparation (0.2 to 1.6 ml), 0.2 mM of DHEA and 0.1 ml of ethyl alcohol. The reaction was started by adding 0.1 ml of the 8 mM alcoholic solution of DHEA. The enzyme reaction lasted for 30 min at 37°C and was stopped by adding 2.0 ml of ethyl alcohol at 0°C . The absorbance of the product, androst-4-ene-3,17-dione, was determined at 248 nm in a Spektromom 403 spectrophotometer. Blank determinations were performed and endogenous values for substrate and protein were subtracted. Specific activity was expressed as μmoles of product per mg of protein per hour. One unit was defined as the amount of enzyme which transforms 1 μmole of DHEA in one hour. Isomerase activity was determined by the method of KAWAHARA *et al.* [3] using androst-5-ene-3,17-dione as substrate. This activity was 25-fold of the oxidoreductase activity, isomerase activity being defined as the amount of enzyme which transforms 1 μmole of androst-5-ene-3,17-dione in one hour.

Protein concentrations were determined according to LOWRY *et al.* [4]. E_{280} values were determined by column chromatography. The method of LISBOA [5] was used for the identification of steroids. Androst-5-ene-3,17-dione was prepared from DHEA by the method of DJERASSI *et al.* [6]. All the other chemicals were commercial products of the highest available grade.

Purification of the enzyme. High losses of activity were encountered when fractionating the cell-free extract with $(\text{NH}_4)_2\text{SO}_4$. Therefore, all the protein

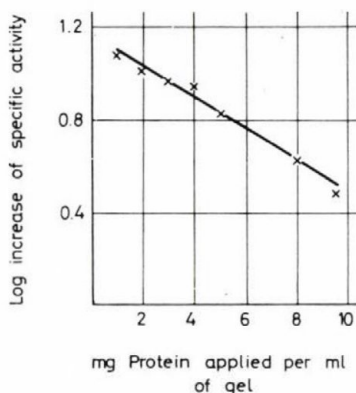


Fig. 1. Increase in specific activity of the enzyme purified on QAE-Sephadex A-50. Column: 1.5×20.5 cm. Flow rate: 10.5 ml/hr. Protein concentration of samples: 25 mg/ml

was precipitated and then a substantial portion of the contaminants was removed by centrifugation. "Negative chromatography" on QAE-Sephadex A-50 proved to be the most efficient way of fractionation. The specific activity was increased best by applying small amounts of sample on the column (Fig. 1). In routine preparative work, however, 5 mg of protein was applied to 1 ml of the gel (Table I).

Table I
Purification of the enzyme

Preparation	Total protein (mg)	Total activity (units)	Specific activity
Cell-free extract	1520	30 400	20
$(\text{NH}_4)_2\text{SO}_4$ concentrate	820	23 780	29
Desalted concentrate	780	22 620	29
Eluent from QAE-Sephadex A-50	84.2	16 400	195
Ficoll concentrate	84.2	14 200	169
Eluent from Sephadex G-100	7.82	13 800	1762

Some properties of the enzyme. In Fig. 2 a typical elution diagram of the $(\text{NH}_4)_2\text{SO}_4$ concentrate from QAE-Sephadex A-50 is illustrated. No separation of oxidoreductase and isomerase is seen. Fig. 3 is a characteristic elution diagram of the Ficoll concentrate from Sephadex G-100. No separation of the two activities is seen here, either. A small part of the activity is excluded from the gel (as well as from Sephadex G-200, thus it is assumed that its $M > 800\,000$), while the greatest part thereof appears in the fractionation range. Assuming the enzyme to be a globular protein, its molecular weight is calculated to be 32 000

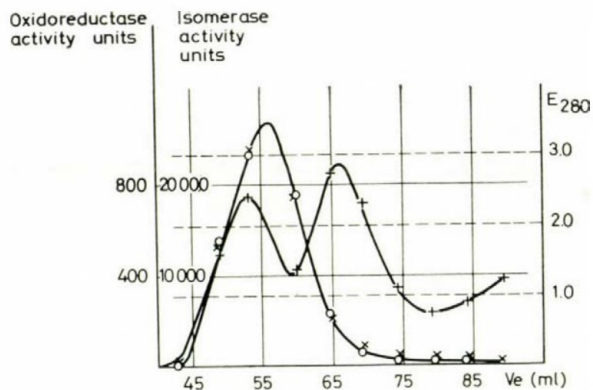


Fig. 2. Purification of $(\text{NH}_4)_2\text{SO}_4$ concentrate on QAE-Sephadex A-50. Column: 2.5×28.5 cm. Sample: 650 mg protein (specific activity: 30, vol.: 22.5 ml). Flow rate: 35 ml/hr. Symbols: $\times$ — $\times$ oxidoreductase activity; $\circ$ — $\circ$ isomerase activity; +—+ E_{280}

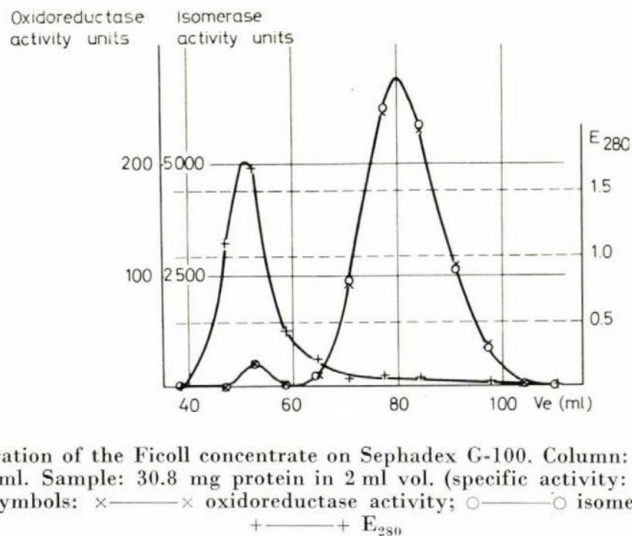


Fig. 3. Gel filtration of the Ficoll concentrate on Sephadex G-100. Column: 2.5 cm. Vol. (blue dextran): 52.5 ml. Sample: 30.8 mg protein in 2 ml vol. (specific activity: 161). Flow rate: 41 ml/hr. Symbols: $\times$ — $\times$ oxidoreductase activity; $\circ$ — $\circ$ isomerase activity; +—+ E_{280}

to 36 000 on the basis of gel filtration on Sephadex G-75 to G-200. The enzyme of high molecular weight was found to be about 15 times more stable than the smaller one at 28°C and 37°C . Enzyme activity is rapidly and irreversibly lost during the course of gel filtration in the absence of magnesium ions (hence the Tris buffer used for purification contained 5 mM MgCl_2). Repeated gel filtration of the excluded activity yielded the same relative amount of small enzyme molecules as did the former operation. The degree of dissociation was not influenced by a tenfold change in the flow rate, or by previous sonication of the sample.

Heat inactivation of the enzyme. No activity is lost after 10 cycles of freezing and thawing (storage at -14°C). A quarter of the activity was lost in one

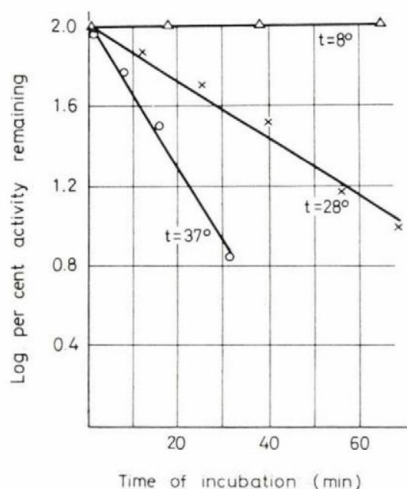


Fig. 4. Temperature inactivation curves of the enzyme at pH 7.15. Assay with androst-5-ene-3,17-dione

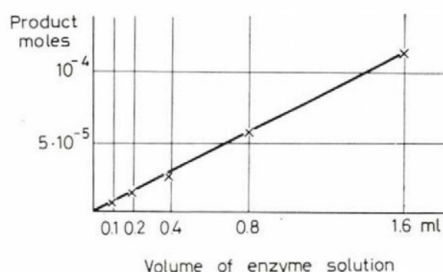


Fig. 5. Initial reaction rate of DHEA transformation. Enzyme: 0.38 mg/ml protein. Specific activity: 1503

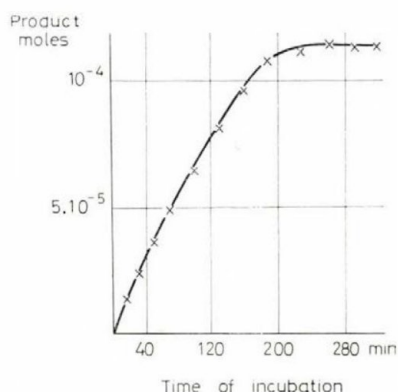


Fig. 6. Effect of time on the formation of androst-4-ene-3,17-dione. The enzyme used would transform the DHEA present in 6 hr

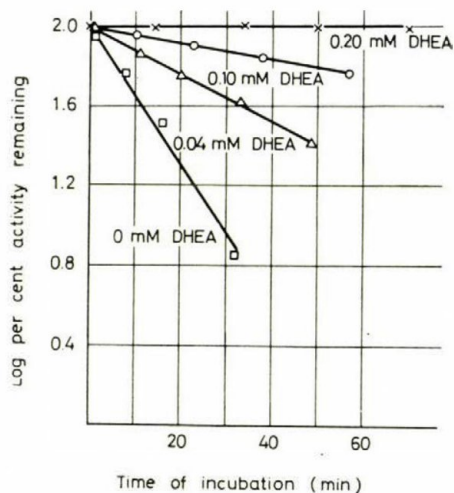


Fig. 7. Protective effect of DHEA against the inactivation of Δ^5 -3 β -hydroxysteroid oxidoreductase at 37°C

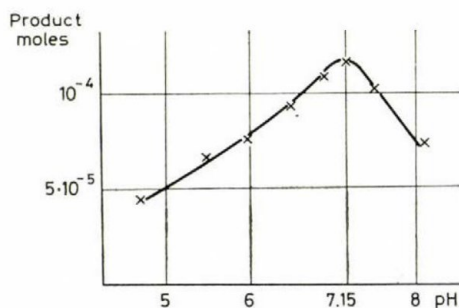


Fig. 8 pH optimum of DHEA transformation in 1 ml 0.1 M Tris buffer containing 0.72 mg/ml protein (specific activity: 1850)

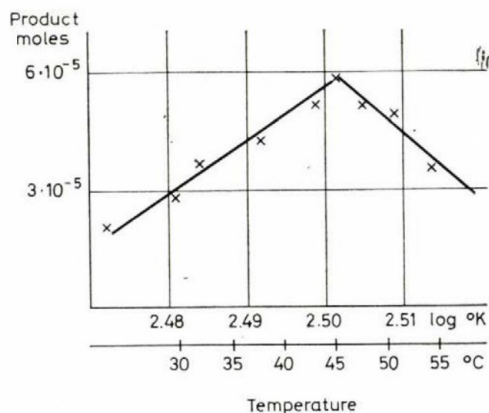


Fig. 9. Temperature of DHEA transformation (specific activity: 1850)

week on storing at 4°C. In Fig. 4 heat inactivation curves of the enzyme are demonstrated. Selective inactivation of the isomerase could be excluded by (a) obtaining the same — although less precise — data with DHEA and (b) obtaining no oxidoreductase activity (DHEA $\rightarrow$ androst-5-ene-3,17-dione transformation, the product yielding androst-4-ene-3,17-dione when NaOH is added) of the enzyme that had been found inactive as an isomerase. It is thus seen that the isomerase activity could not be separated from the oxidoreductase activity, either by selective heat inactivation or by chromatography.

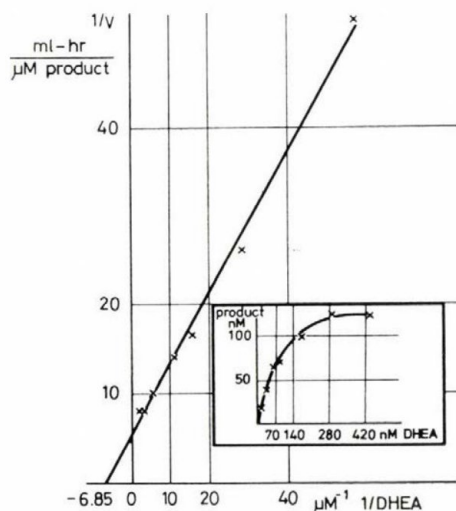


Fig. 10. Lineweaver-Burk plot of DHEA saturation curve. Incubation mixtures contained 912 U

Changing the pH to 5.20 or 8.65 reduced the half time of thermal inactivation at 28°C by about 50%.

The enzymatic reaction. Fig. 5 shows the linearity of the assay with regard to enzyme concentration under the conditions described. Fig. 6 demonstrates that DHEA transformation is linear for about 80 min. This is in apparent contradiction to the fast heat inactivation at 37°C, but on increasing the substrate concentration a marked protective effect was detectable (Fig. 7). No inhibitory effect of the product was noted. The activity was not influenced by p-chloromercuric benzoate or iodoacetic acid. Mercaptoethanol did not protect the enzyme from heat inactivation. The activity was lost on contact with cellulose (ion exchange chromatography, paper electrophoresis).

The pH optimum of the reaction was found at 7.15 (Fig. 8), while the temperature optimum at 45°C, with the plot corresponding to Arrhenius' equation (Fig. 9). The minimum amount of ethyl alcohol required for solubilizing the steroid had no marked inactivating effect on the enzyme.

The above findings justify the conditions of enzyme assay with DHEA with the exception of the temperature of 37°C, where the activity was only 75% of the maximum, but this temperature was easier to maintain. Fig. 10 shows the effect of DHEA concentration on the reaction velocity. K_m was estimated at 1.5×10^{-4} M.

Isomerization of Δ^5 -3-ketosteroids, and notably that of androst-5-ene-3,17-dione, is also an enzymatic reaction. The rate of spontaneous isomerization was increased about 10 000-fold when 0.1 mg of the purest enzyme preparation was present, and the solution accelerating the isomerization was seen to lose its activity under the effect of heat (50°C, 15 min) or in the presence of $(\text{NH}_4)_2\text{SO}_4$.

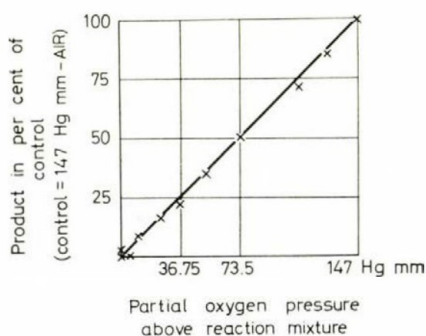


Fig. 11. Dependence of DHEA transformation rate on P_{O_2} in the gas phase under standard conditions. Enzyme: 72 U. Specific activity: 1850. Before starting the reaction, 100 ml of N_2 containing 0 to 21% O_2 was bubbled through the reaction mixture and the substrate solution at 0°C (vol. of gas phase: 10 ml)

NAD, NADP, FMN or FAD did not influence the activity, there having been no change in absorbance at 340 nm, pyridine nucleotides were undetectable in the tryptic-chymotryptic digest of the heat inactivated enzyme, and there occurred no inhibition by acridine or tripaflavine.

No reaction occurred under anaerobic conditions, even in the presence of equimolecular quantities of the above co-factors. In an airtight system, the reaction rate was directly proportional to the partial oxygen pressure in the gas phase (Fig. 11).

Substrate specificity of the enzyme. The transformation of DHEA into androst-4-ene-3,17-dione was irreversible. The enzyme oxidized the 3β -hydroxyl group of steroids also when the A : B ring junction is *trans* in the absence of the Δ^5 double bond. No reaction occurred with a 3-hydroxysteroid with *cis* A : B ring fusion. The enzyme did not oxidize 17β -hydroxysteroids, nor reduce 17-ketosteroids. From among the Δ^5 - 3β -hydroxysteroids with different substituents on ring D, cholesterol was found to be the best substrate as shown by

Fig. 12. The presence of a 20-keto group was advantageous. DHEA with a 17-keto group was also a good substrate. The reduction of this keto group to 17 β -hydroxyl considerably decreased the reaction velocity. Substituents on the α side of ring D slightly also decreased the reaction rate.

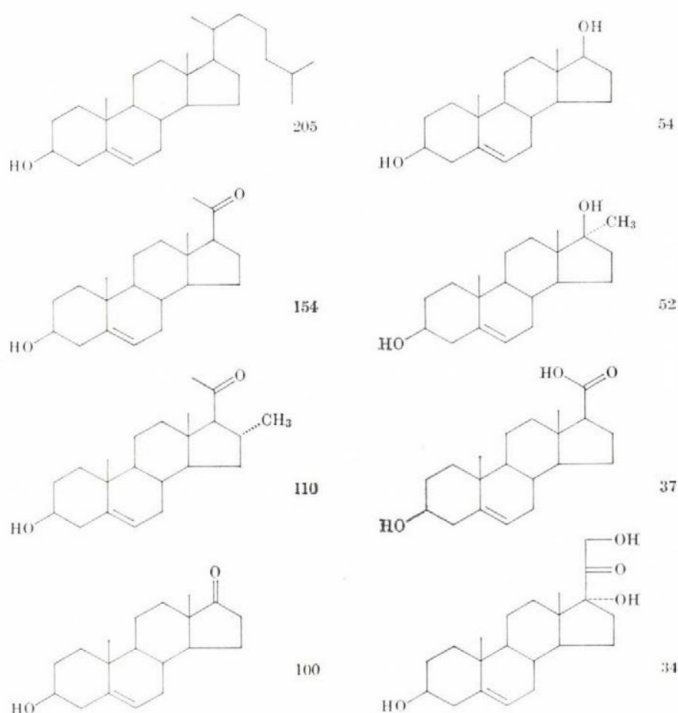


Fig. 12. Effect of substituents on ring D of the substrate on the reaction rate. Relative reaction rate referred to 3 β -hydroxy-5-androsten-17-one equivalent with 100. Differences from standard assay conditions: substrate: 0.06 mM, ethanol: 0.25 ml

Discussion

A new type 3 β -hydroxysteroid dehydrogenase isolated from *S. griseo-carneus* is highly specific for the irreversible oxidation of the 3 β -hydroxyl group in steroids with a *trans* A : B ring junction or a Δ^5 double bond. The inability of the enzyme to oxidize the equatorial hydroxyl group of 5 β -androstane-3 α -ol-17-one indicates a poor contact with the substrate binding site of steroids with a *cis* A : B ring fusion. While the 17 β -hydroxyl group is not oxidized, the data of Fig. 12 indicate that not only ring A but also ring D of the substrate is probably coordinated to the substrate binding site. Although three of the tested substrates were more advantageous, DHEA was routinely used

as a substrate since its water solubility is about ten times better than that of more rapidly transformed steroids and the ethyl alcohol content of the reaction mixture could thus be kept lower. However, in the organism the enzyme is probably present in the form of large molecules ($M > 800\,000$), substrate solubility would hardly play any role *in vivo*.

Molecular oxygen was found to be the hydrogen acceptor in the oxidation. The question of how hydrogen was transmitted to the oxygen remained unclarified, as the role of pyridine or flavine nucleotides could not be detected in the reaction.

Another interesting fact is that the enzyme with a molecular weight of 32 000 to 36 000 also catalyzes the isomerization of the Δ^5 double bond to the Δ^4 position and the two activities cannot be separated. Indeed, it would be surprising to find enzymes with individual binding and active sites and with the summed molecular weight of the above low figure. It is thus suggested that the two reactions are catalyzed by one and the same enzyme in the following manner. The Δ^5 -3 β -hydroxysteroid is bound first and oxidation occurs. Before, however, the product would dissociate, isomerization of the Δ^5 -3-ketosteroid to Δ^4 -3-ketosteroid takes place by virtue of an imidazole residue advantageously oriented in the active site. The two nitrogens of the residue alternately act as proton donor and acceptor, while the 4 β -H is transferred to the 6 β position (*cis* diaxial stereospecificity) as proven by WANG *et al.* [7] and RINGOLD and MALHOTRA [8] for Δ^5 -ketosteroid isomerase. Such a simple enzyme scheme would also seem to be economical for the irreversible transformation of a Δ^5 -3 β -hydroxysteroid substrate into a Δ^4 -3-ketosteroid product.

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STEROID RECEPTORS IN STREPTOMYCES HYDROGENANS: ISOLATION AND CHARACTERIZATION OF A HIGH AFFINITY RECEPTOR FOR 5 α -DIHYDROTESTOSTERONE*

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Summary. There are different steroid binding receptors in the cytosol of *Streptomyces hydrogenans*. A high molecular weight component binds 5 α -dihydrotestosterone (17 β -hydroxy-5 α H-androstan-3-one) with high affinity. Partial purification is achieved by density gradient centrifugation and filtration on Sephadex G-200. 5 α -Dihydrotestosterone and progesterone compete for the same binding sites on the receptor. Cyproterone and testosterone are not bound. As it is easy to isolate the binding fraction, a simple assay for 5 α -dihydrotestosterone or progesterone in biological samples is available.

In higher organisms the biochemical action of steroid hormones starts with the binding of the hormones by specific proteins, which transmit the hormonal signal [1, 2]. In *Streptomyces hydrogenans* (ATCC 19631) steroid hormones stimulate the synthesis and activity of different steroid-metabolizing enzymes. For example the synthesis of 3 α ,20 β -hydroxysteroid-dehydrogenase (EC 1.1.1.53) is increased after culturing the cells in the presence of certain steroids. Most of these steroids are accepted as substrates by the induced enzyme [3]. Additionally, testosterone-17 β -dehydrogenase (EC 1.1.1.63) is activated in the presence of further steroids. Most of these are nearly or completely inactive for the induction of the first-mentioned enzyme [4]. Similar effects on the activity of steroid-metabolizing enzymes have been observed in other microorganisms [5–7].

Because a significant part of the steroids is bound by a high molecular weight fraction of the microorganism *in vivo* [8], we have sought for steroid-binding receptors which are able to bind steroids *in vitro*. Apart from two progesterone-binding fractions a further 5 α -dihydrotestosterone-binding component was found in *S. hydrogenans*. Properties, specificity and abundance of this high molecular weight receptor will be described.

Materials and methods

S. hydrogenans (ATCC 19631) was stored on agar (flaked oats, 3 g; agar, 2 g; NaCl, 250 mg; water, 90 ml; boiled earth-extract [9], 10 ml) at 30°C and cultivated in meat extract medium [3] at 30°C.

* These investigations are part of the inaugural dissertation of J. KURTH, Frankfurt/Main 1974.

After 16 hr the cells were collected by low speed centrifugation and disrupted by sonification in Tris-buffer (10 mM Tris, 1.5 mM Mg^{++} , 10 mM KCl, 1 mM NaN_3 , pH 7.4). After centrifugation of the homogenate at 150 000 g for 3 hr, the supernatant was incubated with 1 mg pancreatic RNase/ml at 18°C for 30 min, and fractionated by gel filtration on Sephadex G-200 (2.6 × 90 cm) at 4°C [8].

The fractions obtained from the Sephadex column were incubated with [3H]5 α -dihydrotestosterone (44 Ci/mmol; NEN New England Nuclear Corp., Mass., U.S.A.) at 4°C for 1 hr. The amount of specifically bound steroid was measured by the dextran-coated charcoal procedure or by rapid filtration on Sephadex G 25 [10]. Both methods gave similar results.

Density gradient centrifugation was done on 10–40% sucrose gradient, running in SW 40 rotor at 40 000 rpm in a Spinco L 2 50B for 4 hr.

Radioactivity of the appropriate samples was measured by liquid scintillation counting in a Packard model 3375. The counting efficiency was 40%.

Results

After sonic homogenization of *S. hydrogenans* the supernatant after centrifugation of the homogenate at 150 000 g (cytosol) was fractionated on Sephadex G-200 (Fig. 1). In the void volume a high molecular weight steroid

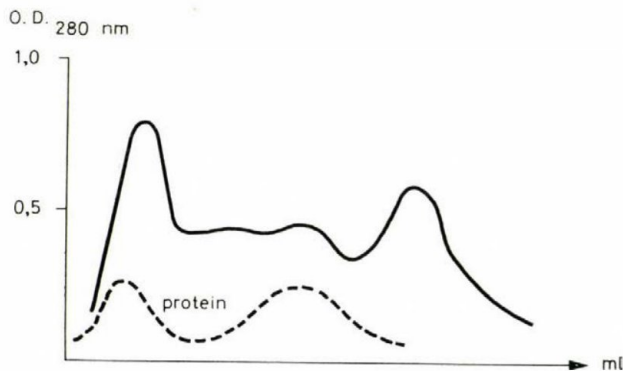


Fig. 1. Gel filtration of 150 000 g supernatant of the cell homogenate of *S. hydrogenans* on Sephadex G-200 (2.6 × 90 cm) with Tris buffer pH 7.4. Optical density at 280 nm (—) and protein content (---) are shown in relation to the elution volume in ml

binding fraction was eluted which bound 5 α -dihydrotestosterone and progesterone with high affinity. This fraction consisted of $\frac{1}{3}$ of the total proteins of the cytosol. To concentrate the binding fraction, ammonium sulphate was added to 50% saturation. The precipitate was dissolved in 5 ml Tris-buffer. Binding activities of the different column eluates were measured after incubation with 10^{-9} M [3H]dihydrotestosterone for 1 hr at least. Unbound and bound steroids were separated by dextran-coated charcoal or by refiltration on Sephadex G-25 (Table I).

The amount of the steroid-binding receptor does not change after cultivation of the cells in the presence of 5 α -dihydrotestosterone *in vivo*. Thus, the steroid-binding activity is not induced *in vivo* by the steroid which

is to be bound. After prolonged cultivation of *Pseudomonas testosteroni* in the presence of testosterone, WATANABE *et al.* [11] found an increase in a testosterone binding protein.

Table I

Binding activity of different cytosols of S. hydrogenans after gel filtration on Sephadex G-200

Cytosol incubated with 10^{-9} M $[7-^3\text{H}]5\alpha$ -dihydrotestosterone (44 Ci/mmmole) for 1 hr. Unspecific binding measured after addition of 10^{-5} M non-radioactive 5α -dihydrotestosterone. Free and bound steroids were separated by the dextran-coated charcoal procedure [8]. The binding index (BI) gives the ratio of specific to total binding

Exp. No.	Protein in the column fraction mg/ml	Total binding cpm/ml	Unspecific binding cpm/ml	BI
1	0.63	24 150	4 450	0.80
2	0.29	2 600	1 970	0.24
3	0.40	7 320	2 000	0.72

The binding protein has a high molecular weight, after gel filtration on a calibrated Sephadex column it is at least 600 000 dalton. Steroid receptors of mammalian organs usually have only molecular weights in the range of 50 000—100 000 dalton [12, 13]. Yet a corticoid-binding receptor with a molecular weight of 620 000 was extracted from mammalian cells [14]. All efforts

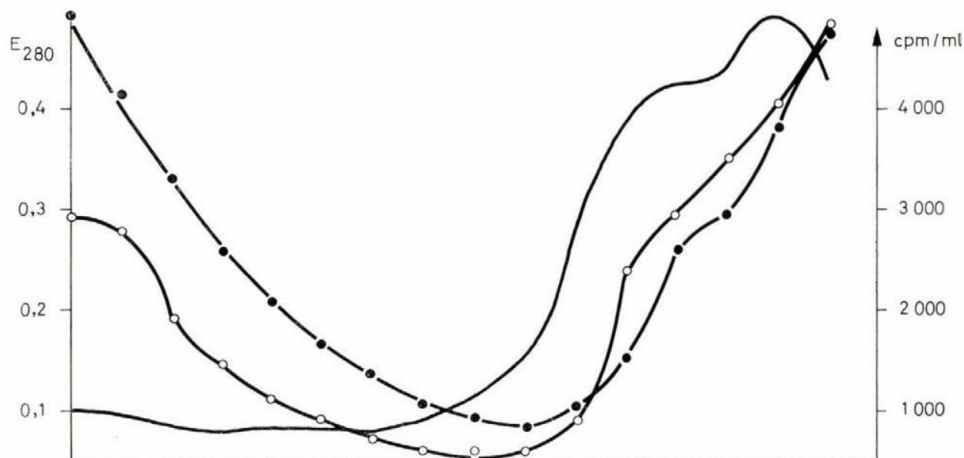


Fig. 2. Sucrose density gradient centrifugation of the high molecular weight fraction after Sephadex G-200 filtration. The high molecular weight fraction was incubated with 10^{-9} M $[7-^3\text{H}]5\alpha$ -dihydrotestosterone (44 Ci/mmmole) at 4°C overnight; 1 ml of the incubate (1.6 mg/ml) was layered on a 10–40% sucrose gradient and run at 40 000 rpm (200 000 g) for 4 hr; 10 drop-fractions were collected after puncturing the bottom of the tube. After addition of 1 ml H_2O , optical density at 280 nm and radioactivity were measured. — optical density (E_{280}); ●—● cpm/ml(10^{-9} M $[^3\text{H}]$ -dihydrotestosterone), ○—○ cpm/ml in the presence of competing dihydrotestosterone (10^{-5} M)

have failed to get a satisfactory purification of the binding fraction of *S. hydrogenans* by ion exchange chromatography on DEAE-cellulose or phosphocellulose. Either the binding fraction was not resolved or it was irreversibly fixed on the column. After centrifugation on a sucrose density gradient (10–40%) the material was running in front of the marker proteins albumin and catalase, proving a very high particle weight of the fraction (Fig. 2).

We suppose that the receptor fraction originates from the cell wall or membranes of the bacteria. If the cells are homogenized under conditions not fragmenting the cell membrane [15], the separated cell wall/membrane fraction behaves like the high molecular weight fraction of the cytosol. Experiments are in progress to split this complex before purification of the receptor by chromatography or electrophoresis. The steroid binding fraction of *Pseudomonas* [11] as well as the corticoid-binding protein from fibroblasts [14] contain lipids which are part of the binding complex.

The 5 α -dihydrotestosterone-binding fraction from *S. hydrogenans* can be inactivated by proteolytic degradation. Incubation with nucleases had, however, no effect (Table II).

Table II

Binding capacity of the high molecular weight fraction of the cytosol of S. hydrogenans after gel filtration on Sephadex G-200

	Binding index (BI)
Control	0.48
+ 1 mg trypsin/ml, 30 min 37 °C	0.22
+ 1 mg RNase/ml, 30 min 37 °C	0.49
+ 0.1 mg DNase/ml, 30 min 37 °C	0.51
+ 10 ⁻⁵ M progesterone	0.15
+ 10 ⁻⁵ M cyproterone	0.50
+ 10 ⁻⁵ M testosterone	0.49

The results showed that dihydrotestosterone-binding sites are able to accept progesterone or 5 α - and 5 β -dihydrotestosterone as well [8]. However, neither testosterone nor the anti-androgen cyproterone compete with dihydrotestosterone. So the high molecular weight fraction of the cells (or even the crude cytosol) may be used to determine the concentration of 5 α -dihydrotestosterone in biological samples. In view of the significant binding of progesterone, this steroid must be removed before the estimation of dihydrotestosterone (Fig. 3).

Figure 3 shows that at least 10⁻⁷ M 5 α -dihydrotestosterone can be detected; this concentration corresponds to 3 ng steroid per ml sample.

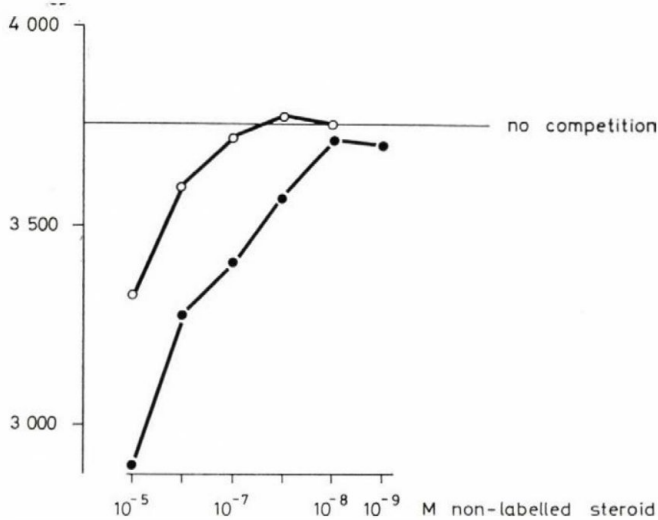


Fig. 3. Estimation of 5 α -dihydrotestosterone by the protein displacement procedure using the high molecular weight fraction of the cytosol of *S. hydrogenans*. 10^{-9} M [$7\text{-}^3\text{H}$]5 α -dihydrotestosterone was added to 0.5 ml of the fraction (1.4 mg protein/ml) and left at 4°C for 1 hr. Further test tubes contained increasing amounts of 5 α -dihydrotestosterone or of progesterone. Binding was stopped by the addition of dextran-coated charcoal. After centrifugation at 1500 g for 5 min, the radioactivity of the supernatant was measured. ●—● 5 α -dihydrotestosterone; ○—○ progesterone

Discussion

The present results show that *S. hydrogenans* contains a steroid-binding fraction, which is able to bind 5 α -dihydrotestosterone or progesterone with high affinity. The association constant is $K_{\text{ass}} = 1.4\text{--}5.5 \times 10^{10} \text{ M}^{-1}$ for progesterone [8] and 10^8 M^{-1} for 5 α -dihydrotestosterone. Because progesterone does not compete as intensely as non-radioactive 5 α -dihydrotestosterone for the dihydrotestosterone-binding sites, we assume that both steroids are bound by different but near or similar binding sites.

All efforts have failed to obtain a pure receptor by chromatography. The best purification was ensured by density gradient centrifugation on a sucrose gradient, proving the high molecular weight of the component. The biological role of the binding activities in *S. hydrogenans* is unknown, and it is also unknown why only 2 out of the 10–15 inducing steroids are bound strongly. There exist perhaps some more special receptors for the further steroids but the amount of these receptors may be too small for detecting them in the cell homogenate. Because there are two enzyme syntheses which are differently regulated by steroids *in vivo* [4], the control system may be more complicated than in typical inductions of catabolic enzymes in bacteria.

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17 β -HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN STREPTOMYCES HYDROGENANS

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Summary. *Streptomyces hydrogenans* converts 17 β -hydroxyandrost-4-ene-3-one (testosterone) to androst-4-ene-3,17-dione (androstenedione) in good yields. Time-dependence of the conversion, steroid uptake and release have been studied *in vivo*. Steroid analysis was done by thin-layer chromatography and recrystallization to constant specific radioactivity. After sonification of the cells the postulated 17 β -hydroxysteroid dehydrogenase activity was recovered in the 105 000 g supernatant. The enzyme was enriched by gel filtration on Sephadex G-200. It required NAD⁺ as cofactor. Its activity could be studied photometrically, because there are no further testosterone-metabolites. If *S. hydrogenans* was cultured in the presence of testosterone, estradiol or 5 α H-dihydrotestosterone, the activity of 17 β -hydroxysteroid dehydrogenase increased.

Enzyme induction of 20 β -hydroxysteroid dehydrogenase (EC 1.1.1.53) is a slower process in the presence of testosterone than in the presence of certain other steroids. It shows a significant time lag [1]. The main conversion of testosterone to androstenedione points to a 17 β -hydroxysteroid dehydrogenase. Since in *Pseudomonas testosteroni* the synthesis of 17 β -hydroxysteroid dehydrogenase and of two other steroid metabolizing enzymes is determined genetically [2], the relationship of 20 β -hydroxysteroid dehydrogenase and 17 β -hydroxysteroid dehydrogenase activity in *Streptomyces hydrogenans* was investigated.

Materials and methods

S. hydrogenans (ATCC 19631) was cultivated, collected after 7 hr and disrupted as described previously [3]. The cell homogenate was centrifuged at 34 500 g to remove the cell walls. The juice was then centrifuged at 105 000 g to obtain the ribosomal fraction (sediment) and the cytosol (supernatant). Cytosol was separated further on Sephadex G-50 column (2.6 $\times$ 35 cm). Radioactivity of the appropriate samples was measured by liquid scintillation counting in a Packard model Tricarb 3375. Counting efficiency was 40%. The pooled steroids of the ribosomal fraction, and the high, medium and low molecular weight cytosols were extracted with ether [4]. Ether extracts were separated on Kieselgel thin-layer plates using cyclohexane:acetic acid ethyl ester (1:2, v/v) as solvent. The metabolites were specified by their R_F -values, UV-fluorescence, dinitrophenylhydrazine reaction and recrystallization to constant specific radioactivity [5]. Added [1,2-³H]testosterone (NEN, Dreieichenhain) allowed quantitation by scintillation counting. Ether extracted radioactivity was regarded as 100%. For the detection of 17 β -hydroxysteroid dehydrogenase, the cytosol was separated by gel filtration on a Sephadex G-200 column [3], and 3 ml fractions were collected; 0.5 mg NAD⁺ in 1.9 ml Tris buffer pH 7.4 and 10 μ Ci [³H]testosterone (final concentration, 8.4×10^{-8} M) in 0.1 ml methanol were added to the fractions to determine their 17 β -hydroxysteroid dehydrogenase activity. *In vitro* samples were incubated at 30°C for 3 hr. Testosterone and androstenedione

were extracted and determined as described before. Enzyme activity was expressed in product/substrate content and not in international units, because detection of the radioactive product demands long incubation periods and there was no substrate saturation.

To increase enzyme activity, 0.34 μ mole of steroid per ml was added to the culture medium. 17 β -Hydroxysteroid dehydrogenase activity was compared in the cytosol by incubation of testosterone *in vitro* as described above.

Results and discussion

S. hydrogenans 105 000 g supernatant separated on Sephadex G-50 yielded a high, a medium and a low molecular weight fraction. One tenth ml of every second tube was quantified in a scintillation counter. Results are shown in Fig. 1.

The distribution of radioactivity after testosterone incubation for 1/4 hr was the same as after 1/2 hr incubation. Radioactivity in the medium cytosol fraction after 1/4 hr and 1/2 hr incubation was less than 0.1% of the total. The biological properties of these two steroid-binding fractions will be investigated later. The high, medium and low molecular weight fractions were pooled, the ribosomal and cytosol fractions were extracted with ether and separated by thin-layer chromatography. Distribution of testosterone and androstenedione was estimated in the ribosomal fraction and in the cytosol (Table I).

Table I

Distribution of testosterone (t) and androst-4-ene-3,17-dione (a) in the ribosomal fraction and in gel-filtered cytosol fractions, in percentage of the total steroids added to Streptomyces hydrogenans during 1/4, 1/2, 1, and 3 hr

Time hr	Ribosomal fraction		High mol. cytosol		Medium mol. cytosol		Low mol. cytosol	
	(t)	(a)	(t)	(a)	(t)	(a)	(t)	(a)
1/4	0.85	0.35	0.15	<0.05	<0.05	<0.05	0.4	0.8
1/2	1.35	0.25	0.05	0.05	<0.05	<0.05	0.9	0.8
1			0.1	0.1	0.05	0.05	0.35	0.65
3	0.1	0.25	<0.05	0.15	0.15	0.05	0.3	0.1

Ribosomal testosterone had a maximum after 1/2 hr, the same as in the low molecular cytosol. Cytosol accepted 1–2% of the steroids added, *i.e.* 1–2 μ g/ml. Testosterone seemed to be pooled in the medium cytosol, while androstenedione in the high molecular weight fraction.

The total steroid content of the cells decreased after 30 min. The values obtained after 30 min incubation of testosterone are of special interest, as RNA-breakdown was observed immediately after the addition of the synthetic corticoid 11 β -21-dihydroxy-pregna-4,17(20)-diene-3-one (dienediol) followed by a faster rate of RNA synthesis that started about 30 min later [1].

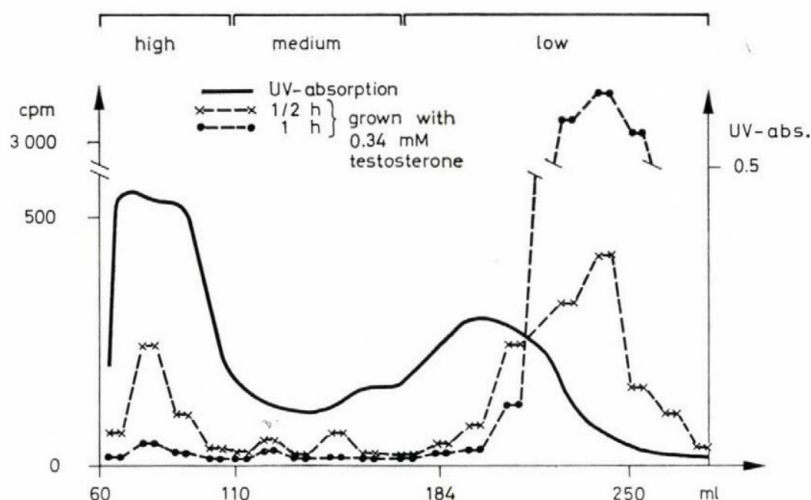


Fig. 1. *S. hydrogenans* 105 000 g supernatant gel-filtered on Sephadex G-50 yielded a high, a medium and a low molecular weight fraction

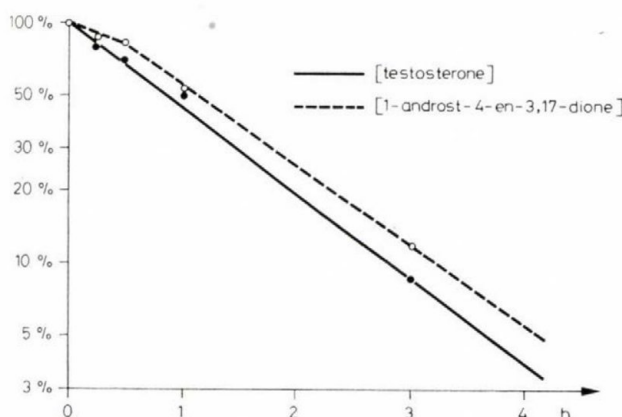


Fig. 2. Time-dependence of the total testosterone content in *S. hydrogenans* culture, and the total androstenedione content

Testosterone conversion in *S. hydrogenans* seemed to follow a first-order reaction, as shown in Fig. 2.

Within 3 hr, *S. hydrogenans* metabolized more than 90 $\mu\text{g/ml}$ of testosterone. Control experiments without *S. hydrogenans* or with isolated cell walls of the microorganism showed that there must be a 17 β -hydroxysteroid dehydrogenase in the 34 500 g supernatant of the cell homogenate. Fig. 3 shows the UV-absorption of cytosol gel-filtered on Sephadex G-200, and 17 β -hydroxy-

steroid dehydrogenase activity of 3 ml fractions in experiments *in vitro*. Enzyme activity is given as quotient Q, the product/substrate content. Molecular weight was about 100 000 dalton.

Thus 17β -hydroxysteroid dehydrogenase was found in the same region as 20β -hydroxysteroid dehydrogenase [6].

If *S. hydrogenans* was cultured in the presence of steroids, some of these increased the enzyme activity. The inducing effect of some steroids on 17β -hydroxy-steroid dehydrogenase can be opposed to that of 20β -hydroxysteroid dehydrogenase of the same microorganism, as shown in Table II.

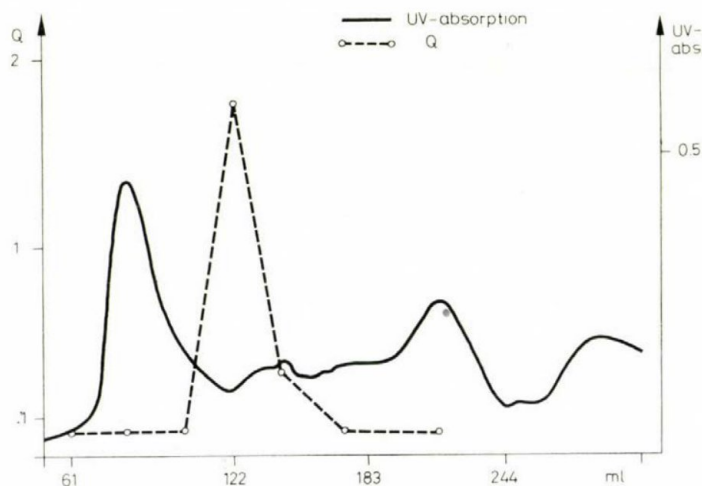


Fig. 3. 105 000 g supernatant of *S. hydrogenans* gel-filtered on Sephadex G-200. Broken line shows the quotient of the relative amount of androst-4-ene-17-dione/testosterone (Q) after incubation *in vitro* of [3 H]testosterone and NAD^+ with 3 ml gel filtration fractions during 3 hr

The first 3 steroids caused an increase of the activity of both enzymes. Cortisol and dienediol had no or only little effect on 17β -hydroxysteroid dehydrogenase but induced a *de novo* synthesis of 20β -hydroxysteroid dehydrogenase. The antiandrogen cyproterone (1,2 α -methylene-6-chloro-17 α -hydroxy-pregna-4,6-diene-3,20-dione) and the estrogen estradiol stimulated 17β -hydroxysteroid dehydrogenase but had no effect whatever on 20β -hydroxysteroid dehydrogenase. Thus, probably these two hydroxysteroid dehydrogenases are regulated differently, whereas 3β -hydroxy- Δ^5 -steroid dehydrogenase showed no activation if the cells were grown in the presence of pregnenolone, a good substrate for this *S. hydrogenans* enzyme [4].

If testosterone was added to the cytosol *in vitro*, no other NAD^+ -linked reaction could be detected in the above experiments. Thus the formation of NADH allowed to measure the testosterone conversion catalyzed by

Table II

Hydroxysteroid dehydrogenase activities after cell growth in the presence of 0.34 μ mole steroid per ml culture medium

Cells grown in the presence of	17 β -Hydroxysteroid dehydrogenase activity in (product/substrate)	20 β -hydroxysteroid dehydrogenase activity in mU/mg protein
testosterone	20	41
α -dihydrotestosterone	5.5	170
pregnenolone	11	170
cortisol	0.6	90
dienediol*	1.5	340
cypoterone	2.8	10
oestradiol	5.5	8
control	0.5	10

* dienediol = 11 β ,21-dihydroxy-pregna-4,17(20)-diene-3-one

17 β -hydroxysteroid dehydrogenase. *Streptomyces hydrogenans* cultured without steroid showed a 17 β -hydroxysteroid dehydrogenase activity of about 50 mU/mg protein as a soluble enzyme.

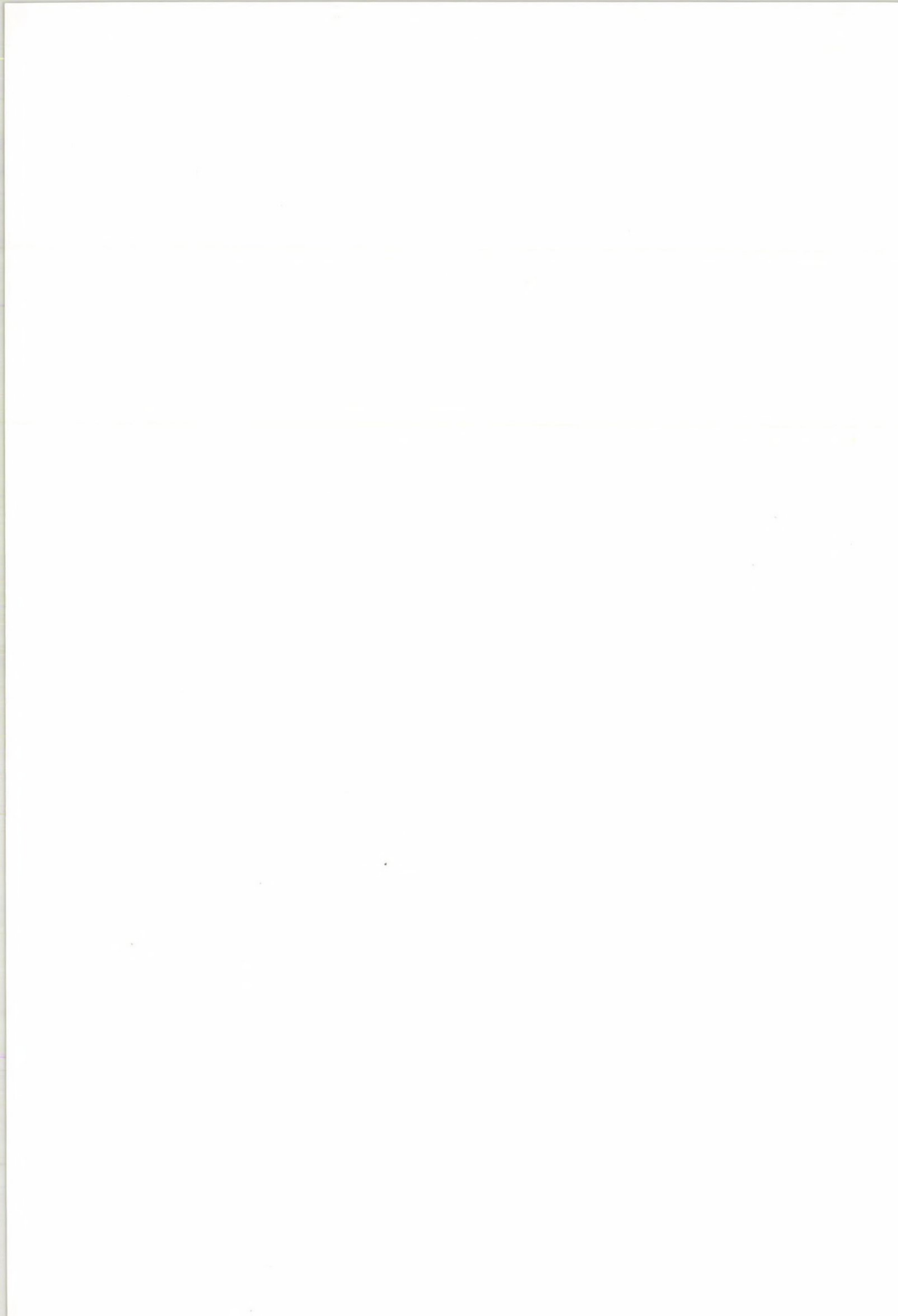
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RNA-METABOLISM IN STREPTOMYCES HYDROGENANS. EFFECT OF 20 β -HYDROXYSTEROID-DEHYDROGENASE INDUCING DIENEDIOL ON RNA-CONTENT AND RNA-PROFILE

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Summary. For the isolation of RNA and polysomes, logarithmically growing cells were disrupted by grinding with kieselgur. Other methods failed to produce undegraded m-RNA as shown by 10–50% sucrose gradient-centrifugation and electrophoresis in 2% mixed agarose-acrylamide gel columns. RNA was extracted by a modified phenol method or in presence of DEP. After the application of dienediol, the amount of acid precipitable RNA decreased by 20 to 50% in 20 min. Incorporation of precursors into RNA was much slower immediately after induction but increased during the next two hours. Three hours after induction there was no difference in the RNA-content of induced and control cultures. Degradation of stable as well as of unstable RNA was observed. Simultaneous addition of the inducer and rifamycin inhibited the production of 20 β -STDH suggesting the synthesis of special m-RNA. The effects of some other antibiotics have also been studied. The existence of a specific m-RNA in the induced system was established from differences in the $^3\text{H}/^{14}\text{C}$ quotients of gel fractions in the region between light r-RNA and t-RNA.

The interrelationship between the biochemical effects of steroid hormones and their physiological action has not been clarified in detail. Concerning sexual hormones, for instance the estrogens, there are data proving the existence of specific receptors [1] which transfer the steroid from the cytoplasm into the nucleus [2]. This is followed by an increase in RNA-polymerase activity [3] and in chromatin template capacity [4]. Therefore the synthesis of new RNA and some new proteins increases [5, 6]. Stimulated r-RNA- and t-RNA-synthesis at the same time and the formation of proteins lead to general tissue hypertrophy.

In *Streptomyces hydrogenans* the formation of a special steroid transforming enzyme, 20 β -STDH, is enhanced by the addition of various steroids to the culture medium [8]. There are specific steroid receptors for the inducer [9] and the activity of RNA-polymerase is also stimulated [10]. Assuming that induction of 20 β -STDH formation followed the same system as that found for other enzyme inductions [11], we have investigated the qualitative and quantitative alterations in the RNA pattern of the microorganism, changes in RNA-content and RNA-profile being primary consequences of the changed cell activities after application of inducing steroids.

Materials and methods

Abbreviations used: 20 β -STDH = 20 β -hydroxysteroid-dehydrogenase; DEP = diethylpyrocarbonate; S-30 = supernatant obtained after centrifugation at a centrifugal force of 30 000 g; S-100 = supernatant obtained after 100 000 g centrifugation; TCA = trichloroacetic acid; dienediol = 11 β ,21-dihydroxy-4,16(20)-pregnadiene-3-one; r-RNA = ribosomal RNA; m-RNA = messenger RNA; t-RNA = transfer RNA; SDS = sodium dodecyl sulphate.

Chemicals. Dienediol was a gift from Dr. G. NESEMAN, Hoechst AG, Frankfurt. DEP, kieselgur and all other substances needed for gel electrophoresis were purchased from Serva, Heidelberg, as well as the antibiotics actinomycin C and rifamycin SV. Fusidic acid was extracted with 95% yield from Fucidine Dragees, Dr. Karl Thomae GmbH, Biberach. [^3H]uracil and [^{14}C]uracil were purchased from NEN, New England Nuclear Co., Dreieichenhain. All other chemicals were of reagent grade and obtained from E. Merck, Darmstadt.

Streptomyces hydrogenans (ATCC 19631) was cultivated and 20 β -STDH activity [8] determined as described previously. Protein content was determined by the method of Lowry, DNA content by the diphenylamine test and total nucleic acids were estimated by the hot TCA-method as described by SOLYMOSY and FEDORCSÁK [12].

Cells were harvested on filter paper under suction and resuspended immediately in ice-cold water, frozen and freeze dried. Dry cells were ground with an equal mass of kieselgur in a mortar [13] and the dry powder was resuspended in RNA extraction buffer: 10 mM Tris, 10 mM NaCl, 1 mM Na-citrate, 1.5% SDS, pH 8, and DEP was added to give a final concentration of 5%. The extraction procedure was as described by SUMMERS [14] and SOLYMOSY *et al.* [15]. Best separation of 10 up to 50 μg of total nucleic acids was achieved by electrophoresis, 150 min at 5 mA/gel, in 2% mixed agarose acrylamide gels as described by BOURQUE and NAYLOR [16]. Following separation the gels were fractionated into 1.4 mm pieces by a Mickle-Gel-Slicer and were subsequently hydrolysed in 0.1 ml of 30% H_2O_2 for 10 hr. No quench effect was measurable. After addition of dioxan, basic scintillator radioactivity was determined in a Packard Tri Carb. Model 3375. For double labelled samples, ^3H efficiency was 25% and ^{14}C efficiency was 54%. ^3H counts were corrected for spillover of ^{14}C counts into ^3H channel, which was 11.2% [17].

Results and discussion

In a number of experiments the optimum extraction procedure for RNA from *S. hydrogenans* was developed. Phenol-extraction modified by ZELLWEGER and BRAUN [18] was compared with the DEP-method. Slight modifications of the above extraction procedures yielded both about 80-90% of total nucleic acids (Table I). RNA extracted with phenol was, however, by far not so pure as that achieved by DEP-extraction (Table II). The more proteins remained with precipitated RNA, the bigger was the chance for a nucleolytic attack by ribonucleases. A second advantage of the DEP-method over the phenol method was that extraction of RNA was achieved within 20 min. Extraction with the phenol method took at least one hour. The amount of extracted DNA was the same with both methods. As there were no further UV-absorbing bands in the OD-profile of a gel except those identified as RNA and DNA, nucleic acids extracted by the DEP method proved of high purity; this was seen in the spectrum, too (Fig. 1).

An essential condition for the quantitative extraction of nucleic acids is an effective and nevertheless careful homogenization of the cells. The homogenizing effect of an X-Press with frozen cells passed through an orifice at 100 kp/cm 2 and of sonication and grinding of freeze-dried cells with kieselgur,

have been compared. As the cell wall of *S. hydrogenans* is not susceptible to lysozyme, protoplasts easily lysed by SDS could not be prepared. As to the quantitative aspect, homogenization by X-Press and sonic power showed optimum results, but the DNA was broken up, r-RNA, when dissociated from

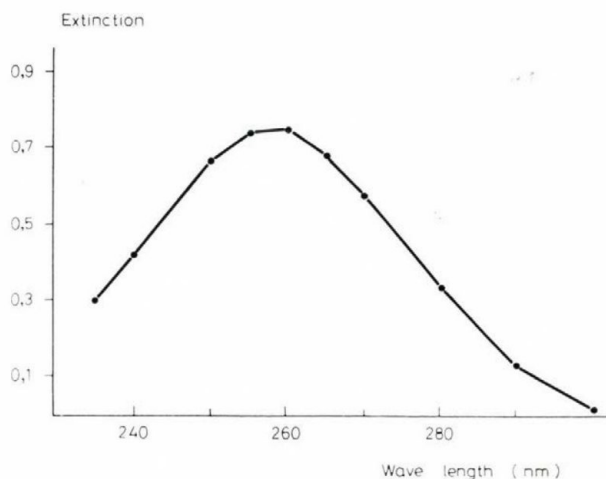


Fig. 1. Spectrum of nucleic acids extracted by DEP-method. Total nucleic acids were extracted as described in Materials and methods and their solution was analysed after appropriate dilution at the given wavelengths

Table I
Recovery of labelled RNA after different extraction procedures

	Radioactivity		Total nucleic acids		DNA mg
	cpm	per cent	mg	per cent	
<i>DEP-extraction</i>					
Cell debris	2.8 × 10 ⁶	12	3.2	7.9	2.5
Alcoholic fraction	0.13 × 10 ⁶	0.5	2.6	6.4	—
RNA-supernatant	20.5 × 10 ⁶	87	34.6	85.0	2.5
Insoluble precipitate	0.14 × 10 ⁶	0.5	0.3	0.7	—
<i>Phenolic extraction</i>					
First phenol phase	0.1 × 10 ⁶	0.4	—	—	—
Second phenol phase	0.2 × 10 ⁶	0.8	—	—	—
Third phenol phase + interphase	5.3 × 10 ⁶	22.4	—	—	—
Alcoholic fraction	0.09 × 10 ⁶	0.4	—	—	—
RNA-supernatant	18.0 × 10 ⁶	76.0	28.0	—	2.5

Cells from a logarithmically growing culture (170 ml) were homogenized and one half was extracted using DEP or phenol. The determined radioactivity was corrected for quenching by the internal standard method. Hot TCA-test for estimation of total nucleic acids failed in samples containing phenol.

its protecting ribosomal particle, was also degraded. X-Press and sonic disintegration could not be used when m-RNA had to be detected in the gels. No rapidly labelled RNA was found in the region between light r-RNA and t-RNA. Because of the high shearing forces during both extraction procedures, the polysomes were torn and at best disomes could be detected in polysome profiles. When polysomes are lacking, m-RNA is fragmented and easily hydrolysed by nucleases. Therefore, polysome profiles obtained after an extraction by means of either X-Press or sonic power looked the same as intact polysomes treated with RNase (Fig. 2).

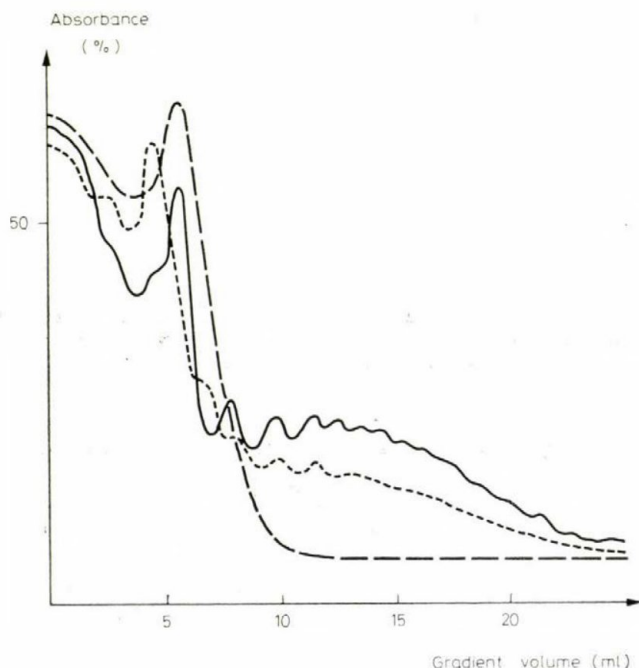


Fig. 2. Polysome profiles obtained after treatment with RNase or EDTA. Cells from the mid log phase were harvested, freeze-dried and ground with kieselgur. The powder was resuspended in polysome buffer: 0.25 M KCl, 10 mM MgCl₂, 5 mM Tris, 0.25 mg fusidic acid per ml, pH 7.2. The supernatant of a 30 000 g centrifugation was used to separate polysomes and subparticles in 15–35% sucrose gradient in a SW-25 rotor at 25 000 rpm for 2 hr. Solid line: untreated polysomes, broken line: RNase-treated polysomes, dotted line: EDTA-treated polysomes

Only grinding of freeze-dried cells yielded qualitatively good and intact nucleic acids as well as polysomes.

Separation of total nucleic acids was best achieved by electrophoresis in 2% mixed agarose acrylamide gel. Separation in 10–50% sucrose gradients and on methylated albumin kieselgur did not possess the resolution power of electrophoresis.

Table II
Amount of macromolecules extracted by DEP or phenol

	Total nucleic acids mg	DNA mg	Protein mg
RNA-supernatant _{DEP}	15.4	3.0	1.15
RNA-supernatant _{phenol}	14.6	2.9	11.05

Total nucleic acids extracted by DEP or phenol were dissolved in buffer and the amount of macromolecules in the solution was determined by spectrophotometry

Following induction of *S. hydrogenans* by dienediol, the cells show a measurable increase in enzyme activity 20 min after addition of the steroid. The increase was nearly linear suggesting a continuous synthesis of specific m-RNA. By the addition of dienediol the rate of cell division, replication of DNA and protein synthesis were not affected. The influence of inducer on RNA content and on RNA synthesis was, on the other hand, dramatic. Within 20 min the amount of RNA decreased by 20 to 50%. The decrease affected not only the nucleotide pool, but also the amount of acid precipitable RNA (Table III).

Table III

Influence of dienediol induction on the amount of nucleic acids and on RNA-synthesis as well as the amounts of DNA, protein and 20 β -hydroxysteroid-dehydrogenase

	Time of cultivation hr	DNA mg/ml	Total nucleic acids mg/ml	Radioactivity		Protein mg/ml	Enzyme mU/mg protein
				total cpm/ml	acid precip. cpm/ml		
Control culture	1	0.112	0.51	17 300	12 700	0.52	15
Induced culture	1	0.116	0.33	9 200	6 600	0.53	210
Control culture	3	0.161	0.61	22 000	19 000	0.84	14
Induced culture	3	0.156	0.48	23 500	18 800	0.87	520

Cells of *S. hydrogenans* were cultivated in the presence of 1 μ Ci/ml [3 H] uracil together with or without dienediol for 1 to 3 hr. DNA, total nucleic acids and radioactivity were determined in the crude homogenate. Protein content and enzyme activity were investigated in the S-100 supernatant.

The RNA content decreased as compared to the control culture and even the rate of RNA synthesis was far lower immediately after induction (Fig. 3). In the second and third hour after addition of the inducer the rate of synthesis increased and after three to four hours the induced cells contained the same amount of RNA as the control cells.

There were strong hints that all species of RNA are degraded, as observed in the double labelling experiments, too (Fig. 5). Rapidly labelled RNA seemed, however, to be degraded preferentially. After cultivation in medium containing radioactive RNA precursors followed by a cold chase in fresh label-free

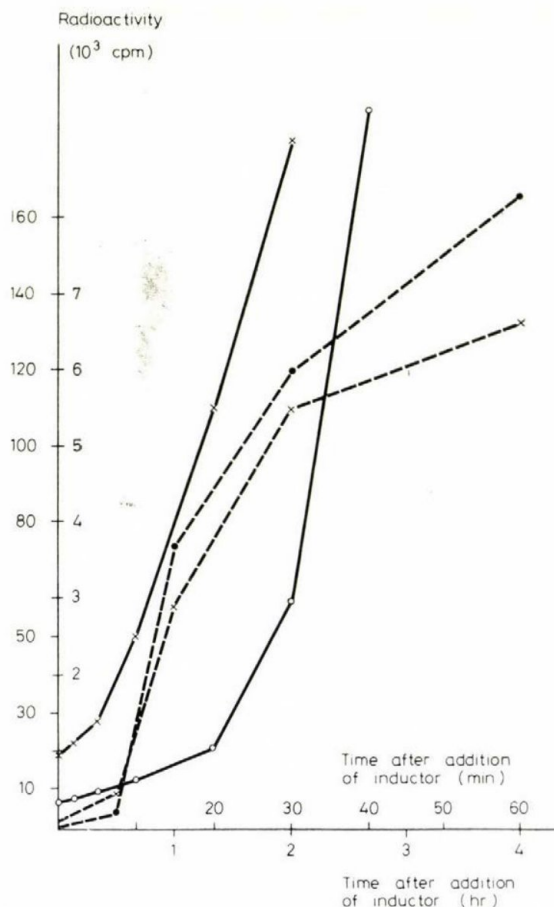


Fig. 3. Kinetics of $[^3\text{H}]$ uracil incorporation. To cells from the mid log phase $1 \mu\text{Ci/ml}$ $[^3\text{H}]$ uracil was added with or without 0.1 mg/ml of dienediol. Control values are given as $\times$, induction values are given as $\circ$. At the times indicated, acid precipitable radioactivity of 1 ml homogenized cell suspension was determined. Solid lines represent the kinetics of $[^3\text{H}]$ uracil incorporation during the first 40 min (inner scale of abscissa and ordinate), broken lines are for kinetics through 4 hr (outer scale of abscissa and ordinate)

medium and simultaneous induction there were far more radioactive degradation products in the medium than in an experiment where induction was started 30 min after the cold chase (Table IV). Using different antibiotics, the relationship between induction of enzyme synthesis and either changed

m-RNA synthesis or favoured initiation of protein synthesis on preexisting long living m-RNA molecules was investigated (Table V). Simultaneous addition of RNA synthesis inhibitor and of inducing steroid allowed no enzyme formation. Without the antibiotic, enzyme activity was significant after 40 min of induction.

Table IV

*Appearance of radioactive degradation products of ^3H -labelled nucleic acids of *S. hydrogenans* in the culture medium*

Experiment	Radioactivity cpm/ml
1 Control culture	1940
2 Induced culture	2680
3 Control culture	2170
4 Induced culture	2140

Four 10 ml cultures, labelled with [^3H]uracil (1 $\mu\text{Ci/ml}$) for 4 hr, were transferred into fresh label-free medium. Cells from experiment 2 were immediately induced adding 0.1 mg/ml dienediol and cells from Experiment 4 were induced after 30 min of cultivation. Radioactivity was determined in 1 ml of cell free medium 30 min after induction.

Table V

Amount of nucleic acids and formation of 20 β -hydroxysteroid-dehydrogenase in the presence of rifamycin SV after 40 min incubation

Dienediol	Rifamycin SV	DNA mg/ml	Total nucleic acids mg/ml	Enzyme mU/mg protein
—	+	0.110 $\pm$ 0.01	0.695 $\pm$ 0.01	7 $\pm$ 2
+	—	0.104 $\pm$ 0.01	0.585 $\pm$ 0.05	121 $\pm$ 10
+	+	0.114 $\pm$ 0.01	0.530 $\pm$ 0.05	5 $\pm$ 1

Four 10 ml cultures were induced by addition of 100 $\mu\text{g/ml}$ dienediol and 1 $\mu\text{g/ml}$ rifamycin SV was added. Amounts of DNA and total nucleic acids were determined from the crude homogenate, enzyme activity was measured in the S-100 supernatant. The figures given are averages of the four cultures.

As shown in Table VI, actinomycin in low doses inhibited only r-RNA synthesis while in higher doses, also the synthesis of m-RNA. After three hours of cultivation and induction in the presence of 0.5 $\mu\text{g/ml}$ actinomycin C, RNA content increased only by 3% as compared to 0 hr value. Despite the strong inhibition of RNA synthesis, a 65% enzyme induction was measured. Fusidic acid blocked the formation of enzyme at a concentration of 0.1 $\mu\text{g/ml}$ completely. RNA synthesis, too, was completely inhibited and even DNA synthesis

Table VI

Influence of different antibiotics on the formation of 20 β -hydroxysteroid-dehydrogenase

Time of cultivation, hr	0	3	3	3	2	3	3	3
Concentration of actinomycin C, $\mu\text{g/ml}$	—	—	0.05	0.1	0.25	0.5	1.0	
DNA, mg/ml	0.22	0.26	0.25	0.27	0.25	0.25	0.25	
Total nucleic acids, mg/ml	0.78	1.34	1.09	0.86	0.85	0.84	0.82	
Protein, mg/ml	—	1.28	1.34	1.28	1.21	0.99	0.95	
Enzyme, mU/mg protein	10	126	111	100	99	81	12	
Time of cultivation, hr	0	3	3	3	3	3	3	3
Concentration of fusidic acid, $\mu\text{g/ml}$	—	—	0.01	0.05	0.1	0.25	0.5	1.0
DNA, mg/ml	0.05	0.1	0.09	0.09	0.07	0.04	0.04	0.03
Total nucleic acids, mg/ml	0.28	0.56	0.69	0.50	0.37	0.24	0.22	0.19
Protein, mg/ml	—	0.74	0.68	0.48	0.42	0.31	0.26	0.20
Enzyme, mU/mg protein	11	193	157	61	9	8	8	8
Time of cultivation, hr	0	3	3	3	3	3	3	3
Concentration of rifamycin SV, $\mu\text{g/ml}$	—	—	0.01	0.05	0.1	0.25	0.5	1.0
DNA, mg/ml	0.138	0.185	0.21	0.20	0.21	0.20	0.20	0.20
Total nucleic acids, mg/ml	0.92	1.165	1.24	1.19	1.19	1.25	1.06	1.09
Protein, mg/ml	—	1.02	1.12	1.15	1.08	1.0	1.24	1.32
Enzyme, mU/mg protein	15	413	425	317	88	34	16	18

Ten ml cultures were harvested prior to induction or supplemented with the above specified amounts of antibiotic and harvested after 3 hr induction.

decreased. Rifamycin had the least influence on RNA synthesis; at a concentration of 1 $\mu\text{g/ml}$ the decrease was 8 %, while 20 β -STDH activity was at the control level. Rifamycin therefore seems to affect m-RNA synthesis preferentially. DNA and protein synthesis were not affected.

Assuming that enzyme induction is based on the synthesis of newly formed m-RNA molecules, this m-RNA must be present in RNA profiles after gel electrophoresis. Rapidly labelled RNA can be found in the gel as a heterogeneous band in the region from 30 S to 5 S (Fig. 4).

Using the double isotope labelling technique, the presence of specific m-RNA in induced cultures was demonstrated on the basis of differences in the $^3\text{H}/^{14}\text{C}$ quotients. Two cultures, one grown in [^3H]uracil-containing medium and one in [^{14}C]uracil-containing medium were pooled prior to extraction of RNA. This RNA was termed RNA_{C0}. Combination of an induced culture grown with [^3H]uracil with one grown with [^{14}C]uracil yielded RNA_{3H}, while the

combination of a culture grown with [^3H]uracil with a [^{14}C]uracil grown and induced culture yielded $\text{RNA}_{14\text{C}}$. One would expect that the mixture of $\text{RNA}_{3\text{H}}$ and $\text{RNA}_{14\text{C}}$ differs from RNA_{C_0} in $^3\text{H}/^{14}\text{C}$ quotient. However, the $^3\text{H}/^{14}\text{C}$ quotient is considerably influenced by the slow rate of RNA synthesis in induced cells. As shown in Fig. 5, [^3H]uracil was incorporated into $\text{RNA}_{3\text{H}}$

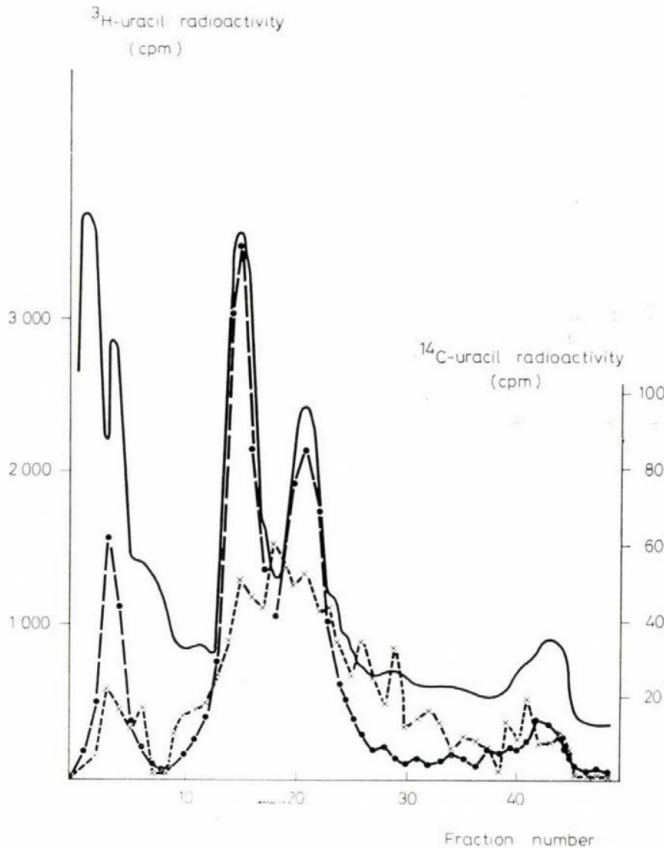


Fig. 4. Pulse labelled RNA in RNA profile after gel electrophoresis. Cells were labelled with 0.05 mCi [^3H]uracil for 3 hr and harvested 10 min after addition of 0.1 mCi [^{14}C]uracil to the medium, freeze-dried and the RNA was extracted. Solid line: OD-profile; broken line: ^3H long term label; dotted line: ^{14}C pulse label

rather than into RNA_{C_0} , while, as expected, [^{14}C]uracil was incorporated in the same amount into both RNA samples.

By division of the gels into five parts and calculation of the average $^3\text{H}/^{14}\text{C}$ quotient it was shown that the relation of the mean r-RNA/t-RNA quotient remains constant in all gels, but the r-RNA/m-RNA quotient is changed. This means that despite the general degradation of RNA in induced

cultures, synthesis of RNA migrating between light r-RNA and t-RNA, which can be termed m-RNA in view of its mobility and rapid labelling, is stimulated by the inducer.

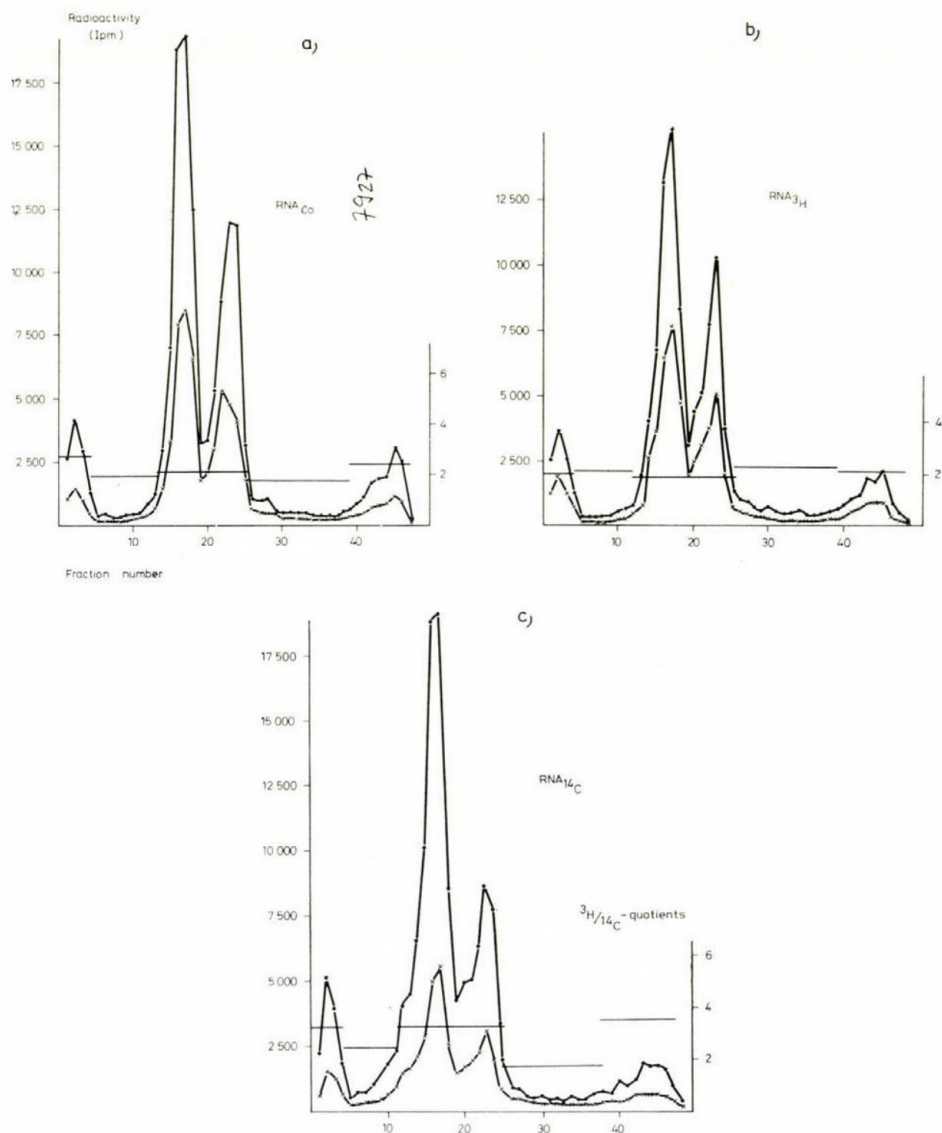


Fig. 5. Demonstration of m-RNA stimulation in induced cells. (a) A cell culture grown with [^3H]uracil was combined with cell culture grown with [^{14}C]uracil. The RNA was extracted, separated by gel electrophoresis and the $^3\text{H}/^{14}\text{C}$ quotient was determined and its mean in the five regions of the gel was calculated. (b) A [^3H]uracil-grown and induced culture was combined with a [^{14}C]uracil-grown culture and treated as described under (a). (c) A [^3H]uracil-grown culture was combined with a [^{14}C]uracil-grown and induced culture and RNA was extracted as described

Thus, formation of 20 β -STDH requires specific m-RNA, the synthesis of which can be inhibited by different antibiotics. As described by FIRTEL and LODISH [19] in *Dictyostelium discoideum*, low doses of actinomycin inhibit r-RNA synthesis, and the same has been now found for *S. hydrogenans*. For this, the complete inhibitory concentration was only one third as compared to that for the former. As cell proliferation and DNA replication were unaffected, actinomycin was not so poisonous than in the chick retinas [20].

Fusidic acid is a more potent inhibitor but it also kills the cells. Similar results were presented for rifamycin on *Bacillus subtilis*, where about 95% of the cells were killed in a few minutes [21].

The partial breakdown of the whole RNA population after the addition of dienediol presents an interesting regulatory phenomenon, probably allowing a more effective translation of newly formed specific m-RNA. OTTOLENGHI *et al.* [22] reported higher incorporation rates of the RNA precursors into rat liver after cortisone treatment. In *Escherichia coli* transferred from minimal to complete medium, RNA synthesis increased [23]. At the same time a 50% decrease of the nucleotides in the first minutes was observed. Whether the RNA content of *E. coli* cells was influenced after shift-up has not been studied. It is generally accepted that m-RNA owing to its low content (1–2%) of total RNA [24] cannot be identified in the OD-profile after gel electrophoresis. The existence of m-RNA as a radioactive heterogeneous fraction can be demonstrated only by separation of the rapidly labelled RNA. In double isotope labelling experiments a stimulation of specific m-RNA could be verified.

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CORRIGENDA

In *Báros, S., Szeri, I., Anderlik, P.*: Combined phytohaemagglutinin and dianhydrosulcitol treatment of lymphocytic choriomeningitis virus infection in mice. *Acta microbiol.*

Acad. Sci. hung. **22**, 237—240 (1975)

page 239, legend to Fig. 1 should read:

Fig. 1. Death of LCM virus-infected animals after dianhydrosulcitol and phytohaemagglutinin treatment. — — — LCM; — · — · DAD-LCM; · · · · PHA-LCM; ——— DAD-PHA—
— LCM

In *Sinkovics, J. G.*: Immunology and putative viral aetiology of human sarcomas. *Acta microbiol. Acad. Sci. hung.* **22**, 223—224 (1975)

page 224, line 6 from the bottom; the sentence beginning "When augmentation . . ." should read:

When augmentation by human sarcoma cells of spleen foci occurs, a spleen focus-forming virus is extractable from the spleens. The envelope antigen composition of this "recombinant", presumably human-mouse, virus differs from that of the mouse spleen focus-forming virus as measured by virus neutralization using mouse and human sera (Dr. F. GONZALES and Miss S. SCHNEIDER): mouse immune sera neutralize readily the mouse spleen focus-forming virus, whereas human sera from sarcoma-bearing patients neutralize the "recombinant" virus but not the mouse virus.

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Ökologie und epidemiologische Bedeutung der infektiösen Chemotherapeutikaresistenz

Von Dr. HELMUT TSCHÄPE, Wernigerode,
und Doz. Dr. HELMUT RISCHE, Wernigerode
(Beiträge zur Hygiene und Epidemiologie, Band 22)

1974. 102 Seiten mit 22 Abbildungen und 23 Tabellen

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Die massenhafte Anwendung der Chemotherapeutika (Antibiotika) bei der industriemäßigen Tier- und Pflanzenproduktion für nutritive, therapeutische und prophylaktische Zwecke sowie in der Humanmedizin für therapeutische Zwecke fördert die Verbreitung der infektiösen Chemotherapeutikaresistenz. Dadurch wird die Chemotherapie der bakteriellen Infektionskrankheiten bei Mensch und Tier zunehmend wirkungsloser. Es werden der derzeitige Stand unserer Kenntnisse über die Ökologie und epidemiologische Bedeutung der infektiösen Resistenz dargestellt und Schlußfolgerungen für ein Überwachungsprogramm gezogen.

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Mykobakterien

Biochemie und biochemische Differenzierung

Von Dr.-Ing. habil. HEINZ IWAINSKY, Berlin-Buch,

und Dr. rer. nat. WOLFGANG KÄPPLER, Berlin-Buch

(Bibliothek für das Gesamtgebiet der Lungenkrankheiten. Bd. 103)

1974. 270 Seiten, 98 Abbildungen, 59 Tabellen

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Gestützt auf die Fülle des Materials des letzten Jahrzehnts werden Zusammenhang, Stoffwechsel und Variabilität von Mykobakterien dargestellt und daraus die potentiellen Möglichkeiten für ihre biochemische Differenzierung abgeleitet und den bei diagnostischen Untersuchungen erhaltenen Ergebnissen gegenübergestellt. Ein Abwägen der einzelnen Verfahren führt zu Vorschlägen für ein zweckmäßiges und zielgerichtetes Vorgehen bei der Differenzierung, die wegen der auftretenden Mykobakteriosen aktuelle Bedeutung erlangt hat.

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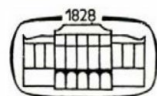
BIOLOGY AND VARIABILITY OF MYCOBACTERIUM TUBERCULOSIS AND THE ATYPICAL MYCOBACTERIA

Experimental and Theoretical Studies

by J. G. WEISZFEILER

This book is a revised and enlarged edition of the author's work which appeared in German, 1969. Relying on his own experimental results and on corresponding literature, the author discusses the sensitivity of mycobacteria to various physico-chemical agents, the problem of their inactivation and reactivation, the hereditary aspects of virulence. New findings in the field of bacterial genetics: plurifactorial mutations and the genetic problems of drug resistance are studied. Results of the investigation of a new vaccine strain "W 115" are submitted and the problems of variability of immunogenicity outlined. The author has discovered two facultatively pathogenic *Mycobacterium* species: *M. simiae* and *M. asiaticum* which might, according to recent research, cause disease in man. As conclusion the principles of evolution of mycobacteria based on darwinism are stressed.

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